INTERACTION OF ADENOSINE RECEPTORS IN A SMOOTH MUSCLE CELL LINE

BY

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1992

This dissertation is dedicated to my grandmother, father, mother and brother.

ACKNOWLEDGEMENTS

First and foremost, I wish to sincerely thank my mentor, Dr. Stephen Baker, for his guidance, both professional and personal, and for the contribution he has made to my education. I would also like to thank Dr. Luiz Belardinelli for his enthusiasm, valuable suggestions and constant supply of wonderful drugs. Many thanks are also expressed to my helpful and friendly committee: Mohan Raizada, Edwin Meyer and Thomas Rowe. I wish to thank all members of Dr. Baker's and Dr. Belardinelli's laboratory, especially Debbie Otero, Dr. John Shryock, Mary Anne Locksmith, Cheryl Spence and Xingmin Tang for their support and technical assistance. I also deeply thanks Drs. Allen Neims, David Silverman, Chingkuang Tu and Thomas Muther for their confidence in me. A special word of thanks goes to Dr. Sandra Rattray for her editorial help. My best wishes are also extended to all my fellow (and former fellow) graduate students especially Sukanya Kanthawatana, Nelida Sjak-Shie, Walter Folger, Daniel Danso, Jiahui Zhang and Magdalena Wozniak; thanks for their friendship and encouragement. I would also like to thank other faculty members who worked so hard to improve the graduate program, and the secretarial and administrative staff who keep the department running. Special thanks are

given to Judy Adams and Barbara Reichert for being so nice to me no matter how many times I bothered them.

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May, 1992

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Major Department: Pharmacology and Therapeutics

DDT_1 MF-2 cells have been shown to express inhibitory A_1 and stimulatory A_2 adenosine receptors (AdoRs) coupled to 3', 5'-cyclic adenosine monophosphate (cAMP) accumulation. The objective of this study was to investigate the possible interaction between the two AdoRs. The AdoR agonists, adenosine and 5'-N-ethylcarboxamido-adenosine (NECA) attenuated isoproterenol (ISO)-stimulated cAMP accumulation in a dose-dependent manner with a maximal inhibition of 68% and 98%, respectively. No cAMP stimulation was observed with either compound. In contrast, the selective A_2-AdoR agonist 2-[2-(2 naphthyl)ethoxy]adenosine (WRC-0018) produced a biphasic response. Stimulation of cAMP accumulation (8-fold) occurred at low concentrations (5 - 500 nM) followed by an attenuation at high concentrations (>500 nM). The attenuation component was prevented by 1) the selective A_1-AdoR

antagonist (±)N^6-endonorbornan-2-yl-9-methyladenine (N-0861, 10 μM), 2) pretreatment of cells with pertussis toxin (PTX, 25 ng/ml, 18 hr) which uncoupled the inhibitory A_1-AdoR response or 3) pretreatment of cells with the selective A_1-AdoR agonist 2-chloro-N^6-cyclopentyladenosine (CCPA, 0.1 μM, 16 hr). CCPA-pretreatment reduced by 13-fold the potency of the A_1-AdoR agonist N^6-cyclopentyladenosine (CPA) to inhibit ISO-stimulated cAMP formation, and decreased the A_1-AdoR level by 48%. Stimulation of cAMP accumulation by adenosine and NECA was uncovered in the presence of N-0861 and by PTX-pretreatment. However, no stimulation by either agonist was observed after CCPA-pretreatment. The data indicate that the inhibitory A_1-AdoR response in DDT_1 MF-2 cells is predominant and masks the A_2-AdoR mediated stimulatory effect. The A_2-AdoR response was expressed by a selective A_2-AdoR agonist or under conditions where the function of the A_1-AdoR is blocked.

The ability of the activated A_1-AdoR to modulate agonist interaction with the beta-adrenoreceptor (βAR) was studied using the irreversible βAR agonist, 5-[2-[[3-[4-(bromoacetamido)phenyl]-2-methylprop-2-yl]amino]-1-hydroxyethyl]-8-hydroxycarbostyril (C-Br). Activation of the A_1-AdoR attenuated cAMP accumulation of the permanently stimulated βAR, and did not alter the irreversible binding of C-Br. In addition, CPA decreased basal cAMP level and had no effect on the interaction of the reversible βAR agonist ISO with the βAR. These data indicate that A_1-AdoR inhibitory

effect is not mediated by alteration of agonist interaction with the ßAR but rather occurs via a post-receptor mechanism.

CHAPTER 1
INTRODUCTION

Functions of Adenosine

History

Drury and Szent-Gyorgyi (1929) were the first to report on the cardiovascular effects of adenosine and adenine nucleotides. They described the isolation of crystalline adenine from acid extracts of ox heart muscle. Adenosine was crystalized from yeast nucleic acid hydrolysate. Intravenous injection of either extract into different mamalian species after atropinization produced primarily sinus bradycardia, transient heart block and other physiological effects (Drury and Szent-Gyorgyi, 1929).

Interestingly, Drury and Szent-Gyorgyi did not comment on the physiological implications of their observations. Within two years, however, Lindner and Rigler (1931) had crystallized adenosine, obtained from the degradation of AMP, from heart muscle extracts. They showed that adenosine was a potent coronary vasodilator in a number of species. Based on the findings that adenosine was present in heart muscle extract and had potent vasoactive effects, Lindner and Rigler advanced the hypothesis that adenosine is a physiological

regulator of coronary blood flow. This hypothesis received little immediate attention and interest at the time.

It was not until 1963 that Berne (1963) and Gerlach et al. (1963) independently revived Lindner and Rigler's adenosine hypothesis by demonstrating the release of adenosine catabolites from hypoxic or ischemic heart muscle. The revived hypothesis states that an imbalance between oxygen supply and oxygen demand leads to alterations of the cellular release of adenosine, which in turn changes the contractile state of vascular smooth muscle of the resistance vessels.

In 1970, Sattin and Rall (1970) and Shimizu and Daly (1970) proposed the existence of extracellular adenosine receptors (AdoRs) based on the observation that adenosine and some adenine nucleotides elevate adenosine 3', 5'-cyclic monophosphate (cAMP) in cerebral cortical slices. This effect was competitively inhibited by methylxanthines, caffeine and theophylline. Within a decade, it became clear that there are AdoRs that inhibit as well as others that stimulate adenylyl cyclase (AC) (Londos and Wolff, 1977; Van Calker et al., 1979), constituting a system for the bidirectional control of the catalytic activity of this key enzyme.

Physiological Effects

Adenosine is present in every cell of the human body and exerts a wide spectrum of effects on various tissues and

organs. For example, in the heart, adenosine is a potent coronary vasodilator (Berne, 1963; Gerlach et al., 1963). It has also been shown to depress cardiac activity, e.g., (1) depress sinoatrial and atrioventricular (AV) node activity, (2) reduce atrial contractility, (3) attenuate the stimulatory actions of catecholamines primarily in ventricular myocardium, and (4) depress ventricular automaticity (Belardinelli et al., 1989). These actions characterize adenosine as an endogenous cardioprotective substance whose actions lead to an increase in oxygen supply and decrease in cardiac work. Together, these actions tend to restore the balance between oxygen supply and demand.

In most tissues including skeletal muscle, adenosine is a vasodilator. Thus, intravenous infusion of adenosine causes hypotension. However, the ultimate cardiovascular effects of adenosine *in vivo* depends on the dose, rate and mode of administration, and on the autonomic reflexes triggered as a result of adenosine's direct action (Pelleg and Porter, 1990).

In the kidney, adenosine produces vasoconstriction of the afferent glomerular artery, a decrease in glomerular filtration rate and inhibition of renin release (Pelleg and Porter, 1990). Adenosine is a depressant of the respiratory center and it causes bronchoconstriction (Pelleg and Porter, 1990). However, adenosine also stimulates arterial chemoreceptors (Biaggioni et al., 1991a). Hence, when given intravenously, adenosine causes hyperventilation (Biaggioni

et al., 1991a). In the nervous system, adenosine produces hyperpolarization of neurons resulting in decreased nerve firing. Adenosine also inhibits neurotransmitter release through putative presynaptic inhibitory receptors, both in the brain and in the periphery nervous system. Adenosine inhibits the release of practically all neurotransmitters studied, including norepinephrine, acetylcholine, dopamine, glutamate, aspartate, γ-aminobutyric acid and serotonin. Adenosine also has a central depressor action and has been proposed as an endogenous anticonvulsant (see references in Biaggioni et al., 1991a). In fat cells, adenosine abolishes the breakdown of stored triglycerides to free fatty acids and glycerol (lipolysis) which is induced by adrenergic stimulation. Adenosine can also prevent platelet aggregation (Berne, 1986; Pelleg and Porter, 1990)

Present and Future Therapeutic Uses

Based on its negative dromotropic effect on AV nodal conduction, adenosine was recently approved by the U.S. Food and Drug Administration as an antiarrhythmic drug for the acute management of paroxysmal supraventricular tachycardia involving the AV node (Belardinelli and Lerman, 1990). In addition, the transient AV block caused by adenosine can also be used to unmask underlying atrial activity in other forms of atrial arrhythmias and hence help in the differential diagnosis of arrhythmias (Belardinelli and Lerman, 1990).

Experimental studies mainly in animal models have indicated several potential uses of adenosine agonists and antagonists. The agonists could be used as antiepileptic, analgesic and sedative drugs due to their inhibitory effect on neurotransmission (Pelleg and Porter, 1990).

AdoR antagonists could be used for the relief of AV block associated with acute myocardial infarction. In addition, they accelerate recovery of myocardial contractility during cardioversion (Wesley and Belardinelli, 1989).

Synthesis and Metabolism of Adenosine

Adenosine is a local hormone (or autacoid) rather than a circulating hormone or neurotransmitter. It acts within the same organ(s), perhaps even on the cell(s), that is the site of its production. Unlike neurotransmitters, adenosine can be produced by virtually any cell. Adenosine per se does not appear to be stored in exocytotic vesicles, but rather is produced on demand, much like prostaglandins and leukotrienes. The primary mechanism for the production of adenosine in heart muscle, liver and leukocytes is the dephosphorylation of AMP by a 5'-nucleotidase located on the cell membrane or in the cytosol. Adenosine produced is then released into the interstitial space from the parenchymal cells for receptor interaction. Physiological stimuli that cause inadequate tissue oxygenation (e.g., hypoxia, ischemia,

exercise) greatly increase adenosine production (Olsson and Pearson, 1990).

Adenosine can also arise from ATP which is released and rapidly broken down by ectonucleotidases. ATP is released from nerve endings (where it is stored in vesicles along with biogenic amines or other classical neurotransmitters), from platelets (where it is stored in secretory granules along with ADP), and from cells that are undergoing lysis. These sources of adenosine probably are important under specific circumstances (i.e., at particular synapses or at sites of injury) (Olsson and Pearson, 1990; Bruns, 1990).

Another intracellular source of adenosine is S-adenosyl-homocysteine (SAH), which arises from S-adenosylmethionine. SAH-hydrolase catalyzes the reversible reaction between SAH and adenosine plus homocysteine. Adenosine also tightly binds to SAH-hydrolase. Hence, under basal conditions, the intracellular concentration of free adenosine is probably very low (Delahaba and Cantoni, 1959; Olsson and Pearson, 1990).

Adenosine crosses cell membranes by simple diffusion and and more importantly by facilitated diffusion. Facilitated diffusion is carrier mediated, nonconcentrative and is inhibited by dipyridamole (DIP) (Kolassa et al., 1970), 6-S-(p-nitrobenzyl-thio)inosine (Paterson and Oliver, 1971) and dilazep (Bruns, 1990). The carrier appears to transport other nucleosides, which are competitive inhibitors of adenosine transport. The carrier is also symmetrical, mediating both

the uptake and release of adenosine with identical kinetics (see references in Olsson and Pearson, 1990).

Adenosine is metabolized very rapidly in the blood with a half-life of 0.6 - 10 sec. The principal route of metabolism is deamination to inosine by adenosine deaminase and further degradation of inosine to hypoxanthine, xanthine and eventually to uric acid. Adenosine deaminase can be inhibited by erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and 2'-deoxycoformycin (see references in Bruns, 1990). In addition, adenosine can reenter the nucleotide pool by phosphorylation to adenine nucleotides (See references in Olsson and Pearson, 1990).

Adenosine Receptors

Classification and Charaterization

Adenosine receptors comprise a group of cell surface receptors that mediate the physiological and pharmacological effects of the nucleoside adenosine. At least two distinct subtypes of cell surface AdoRs are responsible for these actions. These receptors have been classified as A_1-AdoRs and A_2-AdoRs based on biochemical and pharmacological criteria, i.e., modulation of adenylyl cyclase (AC) and differential selectivity for a series of adenosine analogs. The A_1-AdoR that mediates the inhibition of AC has an agonist potency series of R-phenylisopropyladenosine ((R)-PIA) > 5'-N-ethylcarboxamide adenosine (NECA) > (S)-PIA. The A_2-AdoR that

mediates the stimulation of AC has an agonist potency series of NECA > (R)-PIA > (S)-PIA (Van Calker et al., 1979; Londos et al., 1980). The A_2-AdoR in brain has been further subdivided into A_{2a} and A_{2b} subclass. Central A_{2a}-AdoRs are localized primarily in the striatum, nucleus accumbens and olfactory tubercle, whereas central A_{2b}-AdoRs are present in all brain regions. Adenosine and NECA have a higher affinity for the A_{2a}-AdoRs than the A_{2b}-AdoRs (Daly et al., 1983; Bruns et al., 1986).

In addition to modulating AC activity, AdoRs are coupled to other effector systems such as K^+ and Ca^{2+} channels and to phospholipid hydrolysis. Electrophysiological studies indicate that adenosine activates an inwardly rectifying potassium current (I_{KAdo}) in sinoatrial (Bellardinelli et al., 1988), atrial (Belardinelli and Isenberg, 1983) and neuronal (Trussel and Jackson, 1985) cells. The activation of I_{KAdo} is mediated via a pertussis toxin-sensitive guanosine triphosphate (GTP)-binding protein (Kurachi et al., 1986). Adenosine also attenuates the activity of the voltage-sensitive Ca^{2+} channels in hippocampal neurons via presynaptic A_1-AdoRs (Schubert, 1985). Adenosine has also been found to indirectly stimulate inositol phosphate accumulation in guinea pig cortex, FRTL-5 thyroid cells and vas deferens. In these tissues, the effect of adenosine is to potentiate the responses of neurotransmitters such as histamine, norepinephrine and angiotensin II. However, neither adenosine nor its analogs alone increase inositol phopholipid

hydrolysis in these tissues (Hill and Kendall, 1987; Hollingsworth and Dally, 1985; Linden, 1991). In contrast to potentiating the stimulatory response of other neurotransmitters on phospholipid metabolism, in several other tissues (eg., mouse cortex, brown fat and GH_3 pituitary cells), activation of A_1-AdoR leads to inhibition of inositol phosphate accumulation (Kendall and Hill, 1988; Delahunty et al., 1988; Linden and Delahunty, 1989; Linden, 1991).

Both A_1- and A_2-AdoRs are widely distributed in the central nervous system and peripheral tissues. For example, A_1-AdoRs are present in the brain, heart, kidney, lung, pancreas and adipocytes and A_2-AdoRs are present in the brain, coronary arteries, kidney and lung (Olsson and Pearson, 1990).

Analysis of structure-activity relationship indicates that certain N-6 substituents of adenosine enhance the potency of adenosine as an A_1-AdoR agonist (Olsson and Pearson, 1990). For example, cyclopentyladenosine (CPA) and 2-chloro-N^6-cyclopentyl-adenosine (CCPA) have $K_i(A_2)/K_i(A_1)$ ratios of 2500 and 9750, respectively (Lohse et al., 1988). Several purines with C-2 substituents (e.g., 2-aralkoxyadenosine, 2-alkoxyadenosine) have increased potency as A_2-AdoR agonists. For instance, 2-[2-(2-naphthyl)ethoxy] adenosine (WRC-0018) is a highly selective A_2-AdoR agonist (Ueeda et al., 1991). Examples of AdoR agonists and antagonists and their chemical structures are shown in Figures 1-1, 1-2.

Radioligand binding studies of AdoRs have been attempted within the past decade and some success has been achieved, particularly with A_1-AdoR ligands. The first successful radioligand binding studies of AdoRs were reported in the early 1980s. Several groups used a variety of tritiated or iodinated radioligands including both agonists and antagonists (Linden et al., 1985; Trost and Schwabe, 1981; Bruns et al., 1980; Williams and Risley, 1980). Radioligand binding studies in membrane preparations from various tissues revealed all the appropriate characteristics; that is 1) saturability, 2) reversibility, 3) stereoselectivity and 4) the pharmacological specificity expected of the physiologically relevant receptor. A recent development in this field has been the synthesis of the high affinity A_1-AdoR selective radioligand [^{3}H]8-cyclopentyl-1,3-dipropyl-xanthine ([^{3}H]CPX) (Bruns et al., 1987). This A_1-AdoR antagonist has a 740-fold A_1-AdoR selectivity over A_2-AdoR, the highest selectivity reported for an adenosine antagonist. CPX also has very high affinity for the A_1-AdoR (K_D = 0.4 nM) and extremely low non-specific binding (=3% of total binding) in rat brain membranes.

Although the availability of agonist and antagonist radioligands has enabled detailed characterization of the A_1-AdoR in various tissues (Stiles et al., 1985; Jacobson et al., 1986; Ramkumar and Stiles, 1988; Martens et al. 1987), the lack of a highly selective A_2-AdoR antagonist has hampered a similar characterization of the A_2-AdoR. Because

[^{3}H]NECA has high affinity for A_{2a}-AdoR, it has been used as a radioligand for this receptor. However, NECA also binds to A_1-AdoR with high affinity. Thus, when [^{3}H]NECA is used, it is necessary to block the A_1-AdoR by adding a highly selective A_1-AdoR ligand such as the agonist CPA or the antagonist CPX (Hutchison et al., 1989; Linden, 1991). Another A_2-AdoR agonist radioligand recently synthesized is 2-[4-(2-{[4-aminophenyl]methylcarbonyl)ethyl)phenyl]ethylamino-5'-N-ethylcarboxamido adenosine ([^{125}I]PAPA-APEC) (Ramkumar et al., 1990). Likewise, 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethyl-carboxamido adenosine (CGS 21680), an agonist with high affinity and selectivity for A_2- over A_1-AdoR has been synthesized (Hutchison et al., 1989; Lupica et al., 1990). Specific binding of the newly synthesized [^{3}H]CGS 21680 to rat striatal membranes was saturable and reversible. Saturation studies revealed that [^{3}H]CGS 21680 binds with high affinity (K_D=16 nM) to a single class of binding sites. Adenosine agonists competed for the binding of [^{3}H]CGS 21680 with the following potency order: CGS 21680 > NECA > (R)-PIA > (S)-PIA. The specific binding of [^{3}H]CGS 21680 was greatest in rat striatal membranes but negligible in rat cortical membranes. These results indicate that [^{3}H]CGS 21680 directly labels the high affinity A_{2a}-AdoR in rat brain without the need to block binding to A_1-AdoRs (Jarvis et al., 1989; Jarvis and Williams, 1989).

A_1-AdoRs of brain, heart, or fat cells, when labeled with photoaffinity ligands or by means of photoaffinity

cross-linking, migrate on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an molecular mass between 35 and 38 kDa (Ramkumar et al., 1990). In comparison with A_1-AdoRs, photolabeled A_2-AdoRs migrate on SDS-PAGE with a M_r of 42,000 (Ramkumar et al., 1990). Both receptors are known to be glycosylated (Ramkumar et al., 1990). Treatment of photoaffinity-radiolabeled A_1-AdoR of brain with an endoglycosidase (Stiles, 1985) or with either trifluoromethanesulfonic acid or α-mannosidase (Klotz and Lohse, 1986) reduces the molecular mass of the ligand-binding peptide to 32 kDa. A role for protein glycosylation in the function and cellular processing of the AdoR is at present unknown. However, glycosylation has been reported to be essential in the synthesis of insulin receptors (Ronnett and Lane, 1981) but not ß-adrenoreceptors (ßAR) (Doss et al., 1985). In addition, glycosylation also appears to be required for the maintenance of cell surface muscarinic receptors (Liles and Nathanson, 1986).

A_1-AdoRs from rat brain membranes were purified in 1989 (Nakata, 1989a,b; Munshi and Linden, 1989). A_1-AdoR was solubilized with digitonin and purified approximately 50,000-fold to apparent homogeneity by two cycles of affinity chromatography using an antagonist affinity column. The purified receptor migrated on SDS-PAGE with M_r=34,000 either in the absence or presence of 2-mercaptoethanol, suggesting that the receptor does not contain disulfide-linked subunits (Nakata, 1989a).

Recently, two previously cloned proteins (RDC7 and RDC8) with deduced seven transmembrane helices have been identified as canine A_1- and A_{2a}-AdoR subtypes, respectively (Libert et al., 1989; Maenhaut et al., 1990). The deduced molecular masses of RDC7 (36,356 Da) and RDC8 (45,008 Da) correspond closely to the apparent molecular masses of A_1- and A_{2a}-AdoRs estimated by photoaffinity labeling. Features shared by both proteins include small N-termini, conserved transmembrane domains and at least one cysteine in exofacial loops 1 and 2. Notable by their absence are consensus sequences for N-linked glycosylation in the N-terminal segments and an aspartate residue in the third transmembrane segment, the hallmark of cationic amine receptors. Clusters of serine and threonine residues in the C-terminal segments, commonly seen in guanine nucleotide-binding protein (G protein)-linked receptors, are absent in RDC7. Finally, at 326 amino acids, RDC7 is among the smallest members of the G-protein-coupled superfamily of receptors. RDC7 and RDC8 are similar to a variety of other superfamily members, but none of these is more than 30% identical to either of the AdoRs. The expression of RDC8 in adrenal cells, thyrocytes and *Xenopus* oocytes resulted in activation of AC in the absence of added AdoR agonist. Membranes from Cos 7 cells transfected with RDC8 cDNA exhibited binding characteristics of an A_2-AdoR. Moreover, RDC8 mRNA and A_2-AdoR displayed a very similar distribution in the brain (Maenhaut et al., 1990). These data all support that RDC8 is an A_2-AdoR. The gene(s) for the low-affinity A_{2b}-

AdoR has yet to be cloned. There is no published report about the binding and functional characteristics of RDC7 in an expression system.

Receptor-Guanine Nucleotide Regulatory Protein Coupling

Adenosine receptors modulate AC activity via G proteins, the stimulatory G_s and the inhibitory G_i for A_2-AdoR and A_1-AdoR, respectively.

The activation of AC by receptors coupled to G_s can be described by the ternary complex model. This model has been developed for the ßAR system and is likely to be applicable for other stimulatory receptors including the A_2-AdoRs. In brief, an agonist (Ag) binds to the receptor (R) to form an Ag-R complex. The affinity of the agonist in the Ag-R complex is relatively low. The complex then undergoes a conformational change and interacts with a G_s protein to form the ternary complex of Ag-R-G_s. The affinity of the agonist in the Ag-R-G_s complex is relatively high. When GTP binds to G_s, Ag-R-G_s is rapidly converted to G_s-GTP and Ag-R. Once formed, G_s-GTP interacts with the catalytic subunit (C) of AC to form the active complex C-G_s-GTP resulting in a conversion of ATP to cAMP. The enzyme activity returns to basal level when guanosine 5'-triphosphatase (GTPase) activity in G_s hydrolyzes the bound GTP to guanosine diphosphate (GDP) with the resultant regeneration of inactive catalytic unit and G_s-GDP. The destabilization of the ternary complex decreases

agonist affinity and increases the dissociation rate (Gilman, 1987).

Several lines of evidence suggest that the AdoR belongs to the class of G-protein-coupled receptors. For A_1-AdoR, GTP and stable GTP analogues decreased the apparent affinity of A_1-AdoR agonists to the receptor in membranes (Goodman et al., 1982; Yeung and Green, 1983; Lohse et al. 1984), solubilized receptors (Gavish et al., 1982; Stiles, 1985; Stroher et al., 1989) and slices of brain tissue (Fastbom and Fredholm, 1990). Treatment with N-ethylmaleimide which uncouples receptors from G proteins inhibited the action of A_1-AdoR agonists without affecting the binding of antagonists (Fredholm et al., 1985). In addition, detergent-solubilized A_1-AdoR co-eluted with a G protein from an agonist affinity chromatography column where GTP or N-ethylmaleimide, agents known to uncouple receptors from G proteins and simultaneously lower the affinity of agonists for the receptor, were used (Munshi and Linden, 1989; Linden, 1991). It was suggested that A_1-AdoR, unlike other G protein coupled receptors, tightly binds to G protein. The coelution did not occur when an antagonist affinity column was used (Nakata, 1989a, b).

For the A_2-AdoR, experiments showed a GTP dependence for NECA stimulation of AC in purified hepatic plasma membranes (Cooper and Londos, 1979). AC activity from other tissues has been shown to be enhanced by or dependent on the presence of a guanine nucleotide (Londos et al., 1979; Fain and Malbon,

1979; Londos, et al., 1981; Wolff et al., 1981). Moreover, adenosine shortened the lag period for the onset of AC activation by Gpp(NH)p (Sevilla et al., 1977; Lad et al., 1980).

The interaction of an activated receptor with G protein is a key step in signal transduction. The regulatory G proteins are heterotrimers with subunits designated α, ß and γ in order of decreasing mass. The functional difference between G_s and G_i resides in the respective α_s and α_i subunits. The structural features common to α_s and α_i include a GTPase activity and regions that recognize and couple these proteins to a receptor, to the ß+γ complex, and to the effector systems. Common ß and γ subunits are functionally interchangable (Gilman, 1987). G proteins cycle between an inactive GDP state and an active GTP state. When GDP is bound, the α subunit associates with the ß and γ subunits to form a $G_{\alpha ß \gamma}$ complex (denoted by G-GDP) that is membrane-bound. When GTP is bound, the α subunit (G_α-GTP) dissociates from the ß and γ subunits ($G_{ß\gamma}$). G_α-GTP released from $G_{ß\gamma}$ then alters the activity of the target, such as AC or an ion channel (Stryer and Bourne, 1986).

Another feature of G proteins is that the cysteine residue in their α subunits is a substrate for adenosine diphosphate (ADP)-ribosylation which transfers an ADP-ribose moiety from nicotinamide-adenine dinucleotide (NAD). G_s is selectively ADP-ribosylated by cholera toxin which in turn inhibits the receptor-stimulated GTPase activity of the G

protein causing GTP to be bound to α_s for a prolonged period. This results in a permanent activation of AC and a large increase in cAMP accumulation (Gilman, 1987; Nathanson, 1987). Pertussis toxin (PTX) selectively ADP-ribosylates G_i (and another guanine nucleotide binding protein, G_o). This covalent modification of G_i inactivates the protein resulting in the loss of receptor mediated inhibition of AC activity (Gilman, 1987; Nathanson, 1987). Thus, these bacterial toxins have been used as an investigative tool to determine the role of G proteins in the action of biologic messengers. For example, studies showed that PTX blocked the ability of A_1-AdoR to inhibit AC (Hazeki and Ui, 1981) and prevented adenosine-induced changes in the rate of beating in rat atria (Endoh et al., 1983).

Mechanisms for Inhibitory Receptor Action

Receptors that mediate the attenuation of AC activity include the A_1-AdoR, muscarinic M_2-acetylcholine receptor, α_2-adrenergic receptor, δ opiate receptor and D_2-dopamine receptor.

Compared to stimulatory receptors, much less is known about the mechanisms whereby inhibitory receptors attenuate the activation of AC by stimulatory receptors. Currently, there is experimental evidence supporting three different models. First, inhibition may occur by preventing the formation of the ternary complex which is composed of the

agonist, the stimulatory receptor and G_s. This results in the inability of the receptor to form a high affinity binding state. Experiments on rat ventricular myocyte membranes showed that PIA inhibited isoproterenol (ISO)-stimulated AC activity (Romano et al., 1988; Romano et al., 1989). This inhibition was antagonized by theophylline. PIA was much less effective at attenuating forskolin-stimulated AC activity and had no effect on 5'-guanyl-imidodiphosphate (Gpp(NH)p)-induced stimulation. In [^{125}I]cyanopindolol (CYP)/ISO competition binding experiments, ISO produced a concentration-dependent displacement of specific [^{125}I]CYP binding with an IC_{50} of 48 nM and Hill slope of 0.6. About 38% of ßARs were in the high affinity state. Gpp(NH)p shifted the competition curve to the right (IC_{50} = 520 nM) and steepened the slope (Hill slope = 1.2) indicating that all of the ßARs were in low affinity state. PIA significantly increased the IC_{50} for ISO in the absence of Gpp(NH)p (IC_{50} = 140 nM) and steepened the slope (Hill slope = 0.9). These findings were interpreted to indicate that binding of ISO to the high affinity state of the ßAR was decreased in the presence of PIA. PIA had no effect on the ISO competition curve in the presence of Gpp(NH)p (Romano et al., 1988; Romano et al., 1989).

The second is the subunit dissociation model. This model is based on the finding that both G_s and G_i share two common subunits, ß and γ, and that α_i is present in excess relative to α_s in most cells. Activation of G_i leads to subunit

dissociation and the release of sufficient quantities of the ß+γ complex. The large increase in the amount of free ß+γ complex in the membrane would disturb the equilibrium which exists between undissociated and dissociated G_s under resting conditions. Thus, by mass action, ß+γ complex would combine with the released stimulatory α subunits preventing its dissociation and subsequent activation of AC. This model, therefore, implies that G_i will only be effective under conditions where the activity of AC is stimulated, i.e., the effectiveness of G_i is related to the level of the dissociated $G_{s\alpha}$ (Morgan, 1989). Experiments supporting this model showed that when platelet membranes were treated for brief periods of time with GTPγS and an α_2-adrenergic agonist in low Mg^{2+} conditions, AC was "irreversibly" inhibited. This inhibition was of the same magnitude as that produced by maximally effective concentrations of ß+γ complex, and it was not additive with the effect of ß+γ. This inhibition is completely overcome by reconstitution of the membranes with physiological concentrations of $G_{i\alpha}$-GDP. The most likely explanation for this observation is the interaction between $G_{i\alpha}$-GDP and $G_{ß+\gamma}$ to relieve the inhibition caused by free ß+γ complex in the membrane (Katada et al., 1984a).

The third is the direct interaction model. It involves inhibition of AC by the released α_i subunits acting directly on the catalytic subunit of the enzyme (Gilman, 1987). Evidence favoring this model over the subunit dissociation model includes the observation that inhibitory agonists are

capable of reducing AC activity in the cyc$^-$ S49 cell mutant. These cells lack $G_{s\alpha}$, and logically, ß+γ is not inhibitory when reconstituted with cyc$^-$ membranes. It was also demonstrated that the isolated $G_{i\alpha}$ from rat liver can inhibit AC activity in membranes from cyc$^-$ S49 cell. The inhibitory effect of $G_{i\alpha}$ was therefore proposed to explain the ability of inhibitory agonists to decrease AC activity in the cyc$^-$ S49 cell mutant (Katada et al., 1984b; Jakobs and Schultz, 1983). This inhibitory effect of $G_{i\alpha}$-GTPγS has also been observed by Roof et al. (1986) in the bovine central nervous system.

Regulation of Adenosine Receptors

Similar to many other receptors, the AdoR appears to undergo desensitization and down-regulation during chronic exposure to an agonist. This effect prevents overstimulation of the receptor. The mechanism(s) of desensitization and down-regulation has/have been studied extensively in the ßAR-AC system and may be applicable to the AdoR. Desensitization describes the phenomenon where an initial exposure of a cell to an agonist results in a reduced capacity of the cell to respond to a second challenge. Two main types of desensitization have been described. "Homologous" desensitization is referred to hormone-specific type where loss of response is only to the activated receptor, whereas other receptor mediated responses remain unaffected.

"Heterologous" desensitization is referred to hormone-nonspecific type where activation of one receptor causes loss of response mediated by other receptors. Desensitization of ßAR system appears to be initiated by receptor phosphorylation which results in functional uncoupling of the ßAR from G_s. Two kinases have been implicated: the cAMP-dependent protein kinase (PKA) which plays a major role in heterologous desensitization; and the cAMP-independent kinase, termed ßAR kinase which specifically phosphorylates the agonist-occupied receptor leading to homologous desensitization. After uncoupling, the receptors appear to be sequestered within the cells. Removal of the agonist after sequestration leads to rapid resensitization of the system. During longer-term agonist treatment, there appears to be a loss of receptors (down-regulation) due to receptor degradation or loss of the recognition site for ligand binding (Harden, 1983).

Studies have shown that *in vitro* exposure of cultured rat adipocytes to (R)-PIA causes concentration- and time-dependent loss of A_1-AdoR and decrease in the content of G_i protein (Green, 1987). These changes were accompanied by attenuation of the antilipolytic effect of (R)-PIA (homologous desensitization) (Green, 1987). In another study, the number of cardiac A_1-AdoRs in chick embryos was decreased by 63% after pretreatment with 1 µM (R)-PIA for 44 hrs (Shryock et al., 1989). Experiments also showed that the desensitization of A_1-AdoR system in DDT_1 MF-2 cells was

accompanied by a decrease in the number of A_1-AdoRs which can form a high affinity agonist binding site and a 3-4 fold increase in the phosphorylation of A_1-AdoR (Ramkumar et al., 1991). Thus, similar to the ßAR system, A_1-AdoR can also undergo desensitization and/or down-regulation after chronic exposure to an agonist. Uncoupling, down-regulation and phosphorylation of the A_1-AdoR may contribute to the desensitization of this inhibitory receptor.

Desensitization of A_2-AdoR-AC system has also been described. Using clonal neuronal cells (NG108-15), which express both A_2-AdoR and prostaglandin E (PGE_1) receptors, PGE pretreatment reduced the effects of both PGE_1 and adenosine to activate AC (heterologous desensitization). In contrast, exposure of NG108-15 to 2-chloroadenosine resulted in a rapid loss of response to 2-chloroadenosine (homologous desensitization), but PGE_1-stimulated AC activity decreased only slightly (Kenimer and Nirenberg, 1981).

Adenosine receptors are regulated during chronic drug treatment with AdoR antagonist or dexamethasone. A recent study showed that exposure of guinea pig myocardium to AdoR antagonist theophylline increased the the number of A_1-AdoR (Wu et al., 1989). In humans, after 7 days of caffeine (AdoR antagonist) abstinence, NECA produced a concentration-dependent inhibition of thrombin-induced platelet aggregation with an EC_{50} value of 69 nM (Biaggioni et al., 1991b). Subjects were then given caffeine 250 mg p.o. 3 times a day for 7 days. Caffeine withdrawal significantly shifted the

concentration response of NECA to the left (EC_{50}=49 nM, $p<0.01$ by ANOVA) indicating sensitization of AdoRs (Biaggioni et al., 1991b). Other examples include pretreatment of DDT_1 MF-2 cells with dexamethasone (Gerwins and Fredholm, 1991). This glucocorticoid caused a concentration- and time-dependent increase in the number of A_1-AdoRs, but did not affect the K_D or the proportion of A_1-AdoRs in high and low affinity states (Gerwins and Fredholm, 1991). (R)-PIA was more potent as an inhibitor of cAMP formation induced by ISO in dexamethasone-treated cells. Addition of glucocorticoid receptor antagonist RU 486 or protein synthesis inhibitor cycloheximide prevented the up-regulation of A_1-AdoR (Gerwins and Fredholm, 1991). In contrast to sensitization of A_1-AdoR-AC system, the A_2-AdoR-AC system was desensitized as indicated by the decreased ability of NECA to increase cAMP formation in dexamethasone-treated cells (Gerwins and Fredholm, 1991).

AdoRs are also regulated under normal and pathophysiological conditions. For example, in the hypothyroid state, (R)-PIA mediated inhibition of AC and its antilipolytic effect is enhanced (Ohisalo and Stouffer, 1979). In contrast to hypothyroidism, the hyperthyroid state is characterized by enhanced lipolytic activity and cAMP accumulation in adipocytes. These are likely related to the loss of inhibitory tone mediated by A_1-AdoR due to a 35% decrease in A_1-AdoR number (Malbon et al., 1978; Rapiejko and Malbon, 1987). Other conditions that may alter AdoRs include

pregnancy, lactation, starvation, obesity and aging (Ramkumar et al., 1988).

Goals

Over the past decade, a great deal has been learned about the pharmacology, biochemistry and physiology of AdoRs. However, much remains unknown. In general, receptors that mediate inhibition of cAMP formation appear to dominate over stimulatory receptors. In the case of AdoRs, if both subtypes are present in a single cell and are simultaneously activated by adenosine, it becomes important to determine under what conditions the A_1- and A_2-AdoR mediated responses will be expressed. Thus, the major goals of this study were 1) to determine pharmacologically if an interaction between A_1- and A_2-AdoR occurs and 2) to define the conditions whereby the expression of the A_2-AdoR mediated response can be demonstrated.

In addition, the hypothesis that the mechanism for A_1-AdoR inhibitory effects involves alteration in the ability of ßAR agonists to interact with the ßAR was investigated. By using an irreversible ßAR agonist that permanently activates the ßAR, it was determined if the resulting response can be modulated by the inhibitory A_1-AdoR.

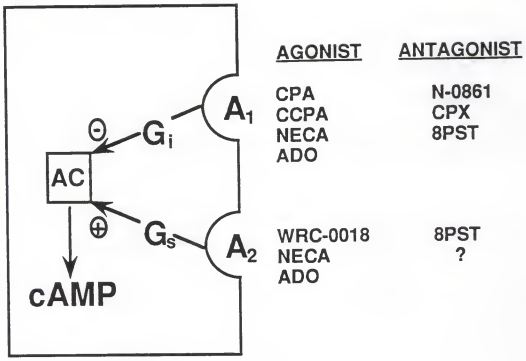

Figure 1-1. Agonists and antagonists of AdoR subtypes.

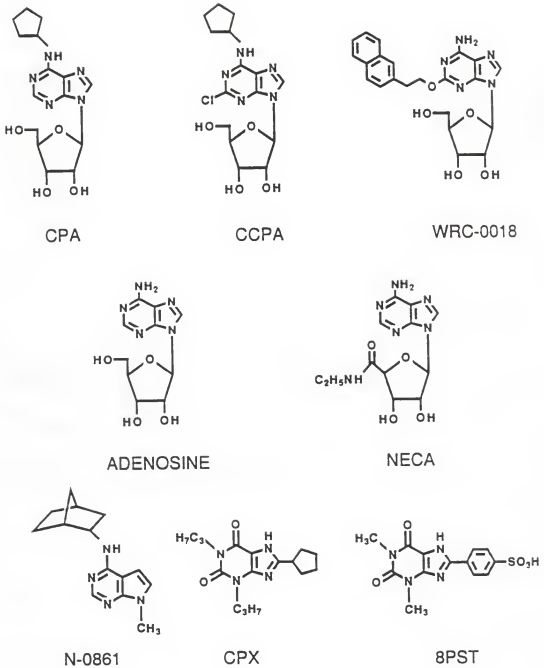

Figure 1-2. Chemical structures of AdoR agonists and antagonists. Abbreviations used: N^6-cyclopentyladenosine (CPA), 2-chloro-N^6-cyclopentyladenosine (CCPA), 2-[2-(2-Naphthyl)ethoxy]adenosine (WRC-0018), 5'-N-ethylcarboxamido-adenosine (NECA), (±)N^6-endonorbornan-2-yl-9-methyladenine (N-0861), 8-cyclopentyl-1,3-dipropylxanthine (CPX), 8(p-sulfophenyl)theophylline (8PST).

CHAPTER 2
EXPERIMENNTAL PROCEDURES

Source of Materials

The radioligands [2,8-^{3}H]adenosine 3', 5'-cyclic monophosphate ([^{3}H]cAMP; 31.2 Ci/mmol), [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]CPX; 99-107 Ci/mmol) and (-)[^{125}I]iodocyanopindolol ([^{125}I]CYP; 2,000-2,200 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA, USA). Adenosine, N^{6}-cyclopentyladenosine (CPA), (-)-N^{6}-(2-phenyl-isopropyl) adenosine ((R)-PIA), dipyridamole (DIP), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), adenosine deaminase (ADA) type VI, 3-[(3-cholamidopropyl)dimethylammoniol]-1-propanesulfonate (CHAPS), benzamidine, (-)-isoproterenol (ISO), 5'-guanylyl-imididodiphosphate (Gpp(NH)p), propranolol (PROP), (±)-alprenolol, penicillin G, streptomycin sulfate, amphotericin B, theophylline, protein kinase, hydroxyapatite and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO, USA). 8-cyclopentyl-1,3-dipropylxanthine (CPX), 8(p-sulfophenyl)theophylline (8PST), 5'-N-ethylcarboxamidoadenosine (NECA) and 2-chloro-N^{6}-cyclopentyladenosine (CCPA) were purchased from Research Biochemicals Inc. (Natick, MA, USA). The DDT_{1} MF-2 (DDT) cell line was obtained from American Type Culture Collection (Rockville, MD, USA).

Dulbecco's modified Eagle's media (DMEM) and fetal bovine serum were from Gibco (Grand Island, NY, USA). Liquiscint was purchased from National Diagnostics (Somerville, NY, USA). 2-[2-(2-Naphthyl)ethoxy]adenosine (WRC-0018) was a kind gift of Dr. Ray. A. Olsson (Univ. of South Florida, Tampa, FL, USA). (±)N^6-endonorbornan-2-yl-9-methyladenine (N-0861) was a gift of Whitby Research, Inc. (Richmond, VA, USA). Pertussis toxin was a gift of Dr. Eric Hewlett (Univ. of Virginia, Charlottesville, VA, USA). Rolipram was a gift of Berlex Laboratories (Cedar Knolls, NJ, USA). 5-[2-[[3-[4-(bromoacetamido)phenyl]-2-methylprop-2-yl]amino]-1-hydroxyethyl]-8-hydroxycarbostyril (C-Br) was synthesized as described previously (Milecki et al., 1987). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) or Fisher Scientific (Orlando, FL, USA).

Methods

Cell Culture

The DDT cell line was derived from a steroid-induced leiomyosarcoma tumor of the vas deferens of an adult Syrian hamster (Norris and Kokler, 1974). These cells were obtained at low passage number and grown as a monolayer on 150 mm plastic culture dishes (Falcon) in DMEM supplemented with 5% fetal bovine serum, 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 2.5 µg/ml amphotericin B in an atmosphere of 5% CO_2/95% air at 37°C. Cells were seeded at 0.2-1 x 10^4

cells/cm^2 and subcultured twice weekly after detachment using 1 mM ethylenediamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS). DDT cells have a doubling time of about 20 hours and a confluent density of 1.3×10^5 cell/cm^2. Experiments were performed on cells 1-day pre-confluent.

Drug Preparation

Stock solutions of WRC-0018 (10 mM) and rolipram (50 mM) were prepared in dimethylsulfoxide (DMSO). CPA (1 mM), CCPA (1 mM), DIP (10 mM) and C-Br (1 mM) were prepared in ethanol. NECA (1 mM) was dissolved in 5 mM HCl and N-0861 (1 mM) was dissolved in a mixture of ethanol (10%) and 50 mM Tris buffer containing 10 mM $MgCl_2$ (90%). These stock solutions were diluted in Hank's Balanced Salt Solution (HBSS) without divalent cations to the desired concentrations just prior to use. HBSS contains 137 mM NaCl, 6 mM D-glucose, 5 mM KCl, 4 mM $NaHCO_3$, 0.6 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$ and 1 mM $CaCl_2$ at pH 7.4. All other drugs were dissolved in HBSS before use.

Drug Treatment

The growth medium in culture dishes was aspirated and fresh medium (20 ml) was then added followed by the drug. The cells were then incubated at 37°C for the various period of times as indicated in the text. At the end of the incubation period, the media was aspirated and the attached cells were

washed four times with 10 ml of ice-cold HBSS without divalent cations.

Membrane Preparation

DDT cells were harvested from culture dishes in 5 ml of 50 mM Tris-HCl buffer at pH 7.4 containing 5 mM $MgCl_2$ with a rubber policeman and were pelleted by centrifugation at 48,000g for 15 min.

For the determination of A_1-AdoR, the pellet was resuspended in ice-cold 50 mM Tris-HCl (pH 7.4) containing 1 mM $MgCl_2$, 1 mM EDTA and 0.1 mM benzamidine (trypsin inhibitor) and then homogenized with a Ten Broeck Tissue Grinder (glass-glass). The homogenate was centrifuged at 48,000g for 15 min to pellet the membranes. The membranes were homogenized a second time in 50 mM Tris-HCl (pH 7.4) containing 1 mM $MgCl_2$, then used for protein measurement and receptor binding assays.

For determination of the ßAR, the pellet from the first centrifugation as described above was homogenized in ice-cold 50 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$ with a Tekmar SDT-100EN homogenizer (setting 5, 20 s). After centrifugation at 48,000g for 15 min and homogenization with the Tekmar as above, the membrane suspension was used for assays.

Protein Measurement

The protein content of cells and membranes was determined by the method of Lowry et al.(1951) using bovine serum albumin as standard.

Radioligand Binding Assay

A_1-AdoRs in DDT cells were determined by specific [^{3}H]CPX binding. Membrane protein was initially incubated with 2 U/ml (1 U = 6.25 µg) ADA for 20 min at 4°C to metabolize endogenous adenosine. Cell membranes (≈0.1 mg protein) were then incubated in a total volume of 0.2 ml with 50 mM Tris-HCl buffer at pH 7.4, 5 mM $MgCl_2$ and 0.06-4 nM [^{3}H]CPX, with or without 50 µM (R)-PIA, for 150 min at room temperature on an orbital shaker. The bound and free ligand were rapidly separated on GF/C glass fiber filters (Whatman Inc., Clifton, NJ, USA) using a Brandel Cell Harvester (Brandel Scientific, Gaithersburg, MD, USA). Filters were rinsed three times with 4 ml of ice-cold 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$ and 0.1 % CHAPS (to reduce non-specific binding). The filters were placed in standard scintillation vials with 10 ml of Liquiscint and the radioactivity was determined in a liquid scintillation counter. Specific binding to A_1-AdoR was calculated as the difference between the total binding in the absence of (R)-PIA and the nonspecific binding in the presence of 50 µM (R)-PIA. Specific binding was generally 90-

95% of total binding. All assays were performed in triplicate, and the determinations differed by less than 6%.

ßARs were quantitated by specific [^{125}I]CYP binding. Membrane protein (30-50 µg) was incubated in a total volume of 0.25 ml with 50 mM Tris-HCl buffer at pH 7.4, 5 mM $MgCl_2$ and 6-100 pM [^{125}I]CYP, with or without 3 µM (±)-alprenolol, for 60 min at 36°C. The bound and free ligand were then rapidly separated on GF/B glass fiber filters using a Brandel Cell Harvester. Filters were rinsed three times with 4 ml of ice-cold 50 mM Tris-HCl buffer containing 5 mM $MgCl_2$, placed in omnivials and the radioactivity was determined in a gamma counter. Specific binding was typically 80-90% of total binding. All assays were performed in triplicate, and the determinations differed by less than 6%.

cAMP Assay

Cells were detached from culture dishes with a cell lifter and centrifuged at 500g for 5 min. The cells (0.4 mg protein/tube ≈ $2x10^6$ cells) were gently resuspended in HBSS containing 50 µM rolipram (phosphodiesterase inhibitor), and incubated at 36°C for 7 min in Beckman microcentrifuge tubes. Drugs were then added and the cells were incubated at 36°C for the various period of times as indicated in the text. At the end of the incubation period, the tubes were immediately placed in a boiling water bath for 5 min. The protein was

pelleted by centrifugation at 9,000g for 2 min, and the supernatants were saved for cAMP assays.

The cAMP content of the supernatant was determined by a modification of a competitive protein binding assay described previously (Baker et al., 1985). An aliquot (usually 50 µl) of the supernatant was incubated in a total volume of 0.2 ml with 25 mM Tris-HCl buffer at pH 7.0, 8 mM theophylline, 0.8 pmol of [^{3}H]cAMP and 24 µg of bovine heart cAMP dependent protein kinase at 4°C for 60 min. At the end of the incubation, 70 µl of a 50% (v/v) hydroxyapatite suspension was added to each tube. The suspensions were then poured onto a Whatman GF/C glass fiber filter under reduced pressure. The filters were rinsed three times with 4 ml of ice-cold 10 mM Tris-HCl buffer and placed in minivials with 3 ml of Liquiscint. Radioactivity was determined in a liquid scintillation counter. The amount of cAMP present was calculated from a standard curve determined using known concentrations of unlabeled cAMP.

Data Analysis

Receptor density (B_{max}) and dissociation constant (K_D) for the radiolabeled ligands were determined from regression analysis of Scatchard plots (1949). The concentrations of compounds which inhibited ligand binding by 50% (IC_{50}) were obtained from Hill plots of the competition data (Hill, 1913). The effective concentrations of drugs which gave 50%

of a maximal response (EC_{50}) were determined using a concentration-effect analysis with a non-linear regression algorithm (Marquardt-Levenberg). Statistical analysis of significance of difference was performed using the Student's t-test.

CHAPTER 3
INTERACTION OF ADENOSINE RECEPTORS

Introduction

Cell surface adenosine receptors (AdoRs) have been classified by pharmacological and biochemical criteria. Two subtypes of AdoRs , i.e., A_1- and A_2-AdoR have been thus far clearly identified. In most cell types studied to date, the A_1-AdoR mediates an inhibition of AC activity whereas the A_2-AdoR mediates a stimulation of the enzyme (Van Calker et al., 1979; Londos et al., 1980). In general, receptors that mediate inhibition of cAMP formation dominate over receptors that mediate stimulation. For example, in mouse atria, carbachol antagonized ISO-stimulated cAMP accumulation by direct activation of the muscarinic receptors. The interaction between carbachol and ISO was not competitive, since cholinergic inhibition could not be surmounted by increasing concentrations of ISO (Brown, 1979). In atria isolated from rats, carbachol decreased the ISO-induced elevation of cAMP levels and inhibited the positive chronotropic and inotropic responses to ISO (Endoh et al., 1985). In addition, after desensitization of the muscarinic system in AtT-20 cells by oxotremorine, cAMP accumulation stimulated by ISO was approximately doubled (Heisler et al.,

1985). In most if not all of these examples, the inhibitory effect cannot be overcome even when the receptor is maximally activated.

The DDT smooth muscle tumor cell line is derived from a steroid-induced leiomyosarcoma of the vas deferens of an adult Syrian hamster (Norris and Kokler, 1974). This cell line has been shown to express a relatively high density of β_2-ARs which mediate a robust stimulation of cAMP formation (Norris et al., 1983). The DDT cells has been used therefore as a model to study this receptor system. These cells have also been shown to express functional histamine H_1 (Mitsuhashi and Payan, 1988) and steroid receptors (Norris and Kohler, 1977). Recently, the presence of both A_1- and A_2-AdoR have been demonstrated in DDT cells by radioligand binding, photoaffinity labeling and their ability to alter AC activity (Ramkumar et al., 1990). In keeping with the classical definition of these two subtypes, the A_1-AdoR in DDT cells inhibits AC activity whereas the A_2-AdoR stimulates the enzyme. The presence of both AdoR subtypes on a single cell coupled to the same second messenger system provides a unique opportunity to characterize pharmacologically if an interaction between the A_1- and A_2-AdoR occurs. Assuming that an A_1-AdoR inhibitory response would mask any A_2-AdoR mediated stimulation of cAMP accumulation, three experimental approaches were used to attempt to alter A_1-AdoR responsiveness and allow the expression of the A_2-AdoR response. These three approaches are shown diagrammatically

in Figure 3-1. First, selective blockade of the A_1-AdoR; second, uncoupling of the A_1-AdoR with PTX and third, down-regulation and/or desensitization of the A_1-AdoR using a selective agonist.

Results

Effects of Selective and Nonselective Adenosine Receptor Agonists on cAMP Accumulation in DDT Cells

Experiments were designed to investigate the effects of the selective A_1-AdoR agonist CPA, the putative nonselective agonists NECA and Adenosine (ADO), and the selective A_2-AdoR agonist WRC-0018 on cAMP accumulation in DDT cells.

Figure 3-2 illustrates the concentration-dependent inhibition of ISO (10 μM)-induced cAMP accumulation by CPA, NECA and ADO in DDT cells. The effect of ADO was studied in the presence of DIP (inhibitor of adenosine transporter) and ENHA (inhibitor of adenosine deaminase) to prevent the uptake and degradation of this nucleoside respectively during the 7 min incubation period. The rank order of potency of these AdoR agonists to inhibit ISO-induced cAMP accumulation was CPA > NECA > ADO with EC_{50} values of 1.5, 14 and 97 nM, respectively. The maximal inhibition of cAMP accumulation caused by the AdoR agonists was 89% for CPA, 97% for NECA and 79% for ADO. Figure 3-3 illustrates the concentration response of ISO to stimulate cAMP accumulation in the absence and presence of CPA (0.1 μM) or NECA (1 μM). These

concentrations of A_1-AdoR agonists caused maximal inhibition of ISO-stimulated cAMP accumulation (Figure 3-2). ISO alone increased cAMP accumulation in a concentration-dependent manner with an EC_{50} of 2.4 nM and a maximal stimulation of 62-fold above the basal level achieved at 0.1 μM. In the presence of CPA or NECA, ISO still stimulated cAMP accumulation in a concentration-dependent manner, but the maximal response was decreased by 75% and 60% in the presence of CPA and NECA, respectively. In the presence of 1 μM CPX, a selective A_1-AdoR antagonist, the inhibitory effect of CPA on ISO-stimulated cAMP accumulation was significantly attenuated (data not shown). Thus, our results indicated that the A_1-AdoR agonists had an inhibitory effect on cAMP accumulation and this effect was mediated by A_1-AdoR.

The effects of the selective A_2-AdoR agonist WRC-0018 (Ueeda et al., 1991) and the nonselective agonist NECA on cAMP accumulation are illustrated in Figure 3-4. WRC-0018 produced a biphasic response whereas NECA caused no effect on cellular cAMP level. WRC-0018 at low concentrations (5 - 500 nM) stimulated cAMP accumulation with a maximal response of 8±1 -fold above the basal level. The estimated EC_{50} was 8.6 nM. However, higher concentrations (>500 nM) of WRC-0018 decreased cAMP accumulation with an EC_{50} value of 5.1 μM. In contrast to WRC-0018, NECA over the entire concentration range of 0.1 nM - 10 μM did not affect (increase or decrease) cellular cAMP content. Similar to NECA, ADO (0.1 nM - 10 μM)

also did not affect the cellular cAMP level above the basal level (data not shown).

Figure 3-5 depicts the effect of the non-selective AdoR antagonist 8PST on the stimulatory effect of WRC-0018. WRC-0018 (0.1 μM) induced a 3-fold increase in cAMP content above the basal level and 8PST (5 μM) inhibited the WRC-0018-induced cAMP accumulation by 80%. Figure 3-6 illustrates a similar effect of the A_1-AdoR agonist CPA on the stimulatory effect of WRC-0018. At a concentration of 0.1 μM, WRC-0018 stimulated cAMP accumulation 3-fold above the basal level and CPA (1 μM) inhibited the WRC-0018-induced cAMP accumulation by 78%.

Figure 3-7 illustrates the effects of WRC-0018 on cAMP accumulation in the absence and presence of 10 μM ISO. The biphasic concentration response curve of WRC-0018 was replotted from Figure 3-4. In the presence of ISO, low concentrations of WRC-0018 (1 nM - 100 nM) did not affect ISO-stimulated cellular cAMP accumulation. However, at higher concentrations (≥500 nM), WRC-0018 attenuated the stimulatory effect of ISO in a concentration-dependent manner with an EC_{50} of 840 nM and a maximal inhibition of 73%.

These data show that nonselective AdoR agonists (NECA and ADO) themselves only inhibit cAMP accumulation. In contrast, the selective A_2-AdoR agonist (WRC-0018) stimulated at lower and inhibited cAMP accumulation at higher concentrations, thereby resulting in a biphasic concentration response curve. To further investigate the expression of the

A_2-AdoR mediated cAMP accumulation, three experimental approaches were used (Figure 3-1). These were 1) selective blockade of A_1-AdoR, 2) uncoupling of A_1 inhibitory effects with PTX, and 3) selective desensitization and/or down-regulation of A_1-AdoR.

Effect of a Selective A_1-AdoR Antagonist on the A_2-AdoR Mediated Response

The first experimental approach used to uncover the A_2-AdoR mediated effect on cAMP accumulation was blockade of the A_1-AdoR with a selective A_1-AdoR antagonist. The ability of the highly selective A_1-AdoR antagonist, N-0861 (Shryock et al., 1992), to compete with [^{3}H]CPX for the A_1-AdoR binding site is shown in Figure 3-8. N-0861 produced a concentration-dependent displacement of specific [^{3}H]CPX binding with an IC_{50} of 0.8 μM and a Hill slope of 1.0. This indicated that N-0861 bound to a single class of binding sites. Figure 3-9 illustrates the effect of N-0861 on the A_1-AdoR inhibitory effect of CPA. ISO (10 μM) produced a 12-fold increase in cAMP accumulation above the basal level and CPA (0.1 μM) inhibited the stimulatory effect of ISO by 70%. N-0861 (10 μM) attenuated the inhibitory effect of CPA by 62%. A similar effect of N-0861 on NECA-induced inhibition of ISO-stimulated cAMP accumulation is shown in Figure 3-10. That is, ISO (10 μM) stimulated cAMP accumulation 47-fold above the basal level and NECA (1 μM) inhibited this increase by 74%. N-0861 (10 μM) attenuated the inhibitory effect of NECA by 60%. The

effect of N-0861 on WRC-0018 stimulation of cAMP accumulation is shown in Figure 3-11. The 3-fold increase in cAMP accumulation caused by 0.1 μM WRC-0018 was not affected by N-0861 (0.1 nM - 10 μM). Based on these results, in the remaining experiments of this series, 10 μM N-0861 was used to selectively block the A_1-AdoR mediated inhibition of cAMP accumulation.

The effects of N-0861 on the A_2-AdoR mediated response of selective and nonselective agonists were investigated. Figure 3-12 illustrates the concentration response of WRC-0018 in the absence and presence of N-0861. The biphasic concentration response curve of WRC-0018 in the absence of N-0861 was replotted from Figure 3-4. N-0861 (10 μM) completely abolished the A_1-AdoR mediated inhibition of cAMP accumulation caused by WRC-0018 and hence, the downward component of the biphasic concentration response curve of WRC-0018 was eliminated. The EC_{50} for the A_2-AdoR mediated effect of WRC-0018 on cAMP accumulation was 93 nM. The maximal stimulation of cAMP accumulation by WRC-0018 was increased ($p \leq 0.05$) from 8±1 -fold in the absence to 15±6 -fold in the presence of N-0861. The effects of NECA on cAMP accumulation in the absence and presence of N-0861 are shown in Figure 3-13. In the absence of N-0861, NECA did not stimulate cAMP accumulation. In contrast, in the presence of N-0861 (10 μM), NECA stimulated cAMP accumulation over the concentration range of 1 μM - 100 μM. At concentrations of NECA ≥10 μM, there was a decrease in the cAMP level despite

the presence of N-0861 in the incubation medium. Similar results to those of NECA were observed with ADO (Figure 3-14). ADO (0.1 nM - 10 μM) caused no effect on the cellular cAMP content in the absence of N-0861 whereas ADO (1 μM - 5 μM) stimulated cAMP accumulation in the presence of 10 μM N-0861. At 10 μM ADO, there was a decrease in cAMP formation despite the presence of N-0861 in the incubation medium.

In summary, these data show that the selective A_1-AdoR antagonist N-0861 abolishes the downward phase of the WRC-0018 biphasic concentration response and uncovers a stimulatory (i.e., A_2-AdoR mediated) response of NECA and ADO.

Effect of PTX on the A_2-AdoR Mediated Response

The second experimental approach used to unmask the A_2-AdoR mediated effect on cAMP accumulation was uncoupling of A_1 inhibitory effects with PTX. Based on a previous study which showed that inhibition of AC activity by (R)-PIA was markedly attenuated after an 18 hr pretreatment of DDT cells with 100 ng/ml PTX (Ramkumar et al., 1990), I chose to incubate our DDT cells with PTX for 18 hr. The ability of CPA to inhibit ISO-stimulated cAMP accumulation in cells pretreated with various concentrations of PTX for 18 hr is shown in Figure 3-15. In control (untreated) DDT cells, 10 μM ISO produced a 15-fold increase in cAMP content above the basal level. As expected, CPA (1 μM) attenuated the

stimulatory effect of ISO by 84%. Pretreatment of cells with ≥ 25 ng/ml PTX for 18 hr resulted in a complete loss of the CPA inhibitory effect on cAMP accumulation. Basal and 10 μM ISO-stimulated cAMP accumulation were not significantly affected after pretreatment of the cells with PTX. Based on these results, in the remaining experiments of this series, DDT cells were pretreated for 18 hr with 25 ng/ml of PTX.

Figure 3-16 depicts the effects of WRC-0018 on cAMP accumulation in control and PTX-pretreated cells. The biphasic WRC-0018 concentration response in control cells was replotted from Figure 3-4. After pretreatment of the cells with PTX, similar to using A_1-AdoR antagonist, the A_1-AdoR mediated inhibition of cAMP accumulation is blocked and hence, the downward component of the concentration response curve of WRC-0018 was abolished. The EC_{50} value of the A_2-AdoR mediated effect of WRC-0018 on cAMP accumulation was 90 nM. The maximal stimulation of cAMP accumulation was 14±3 -fold above basal in PTX-pretreated cells (control cells, 8±1 -fold above basal). The effect of NECA on cAMP accumulation in control and PTX-pretreated cells is shown in Figure 3-17. The data for NECA in control cells were replotted from Figure 3-13. In PTX-pretreated cells, NECA (10 nM - 10 μM) stimulated cAMP accumulation with an EC_{50} value of 180 nM. The maximal response was 5-fold above the basal level. Figure 3-18 illustrates the effect of ADO on cAMP accumulation in control and PTX-pretreated cells. The data for ADO in control cells were replotted from Figure 3-14. Over the concentration range

of 1 μM - 10 μM, ADO increased the cAMP level in PTX-pretreated cells. A maximal response was not achieved even at 10 μM ADO and hence, the EC_{50} value of the A_2-AdoR mediated response of ADO could not be calculated.

In summary, these data show that following PTX-pretreatment, the selective A_2-AdoR agonist WRC-0018 causes a sustained A_2-AdoR mediated stimulatory effect on cAMP accumulation. Likewise, the A_2-AdoR mediated stimulatory effects of the nonselective agonists NECA and ADO were unmasked after pretreatment of DDT cells with PTX. The results also indicate that the A_1 inhibitory effect of AdoR agonists was G_i protein mediated.

Effect of Desensitization and/or Down-regulation of A_1-AdoR on the A_2-AdoR Mediated Response

The third experimental approach used to uncover the A_2-AdoR mediated effect on cAMP accumulation involves desensitization and/or down-regulation of A_1-AdoR. Figure 3-19 illustrates a representative Scatchard plot of [^{3}H]CPX binding to cell membranes from control (untreated) and CCPA (A_1-AdoR agonist)-pretreated cells. The inhibition of ISO (10 μM)-stimulated cAMP accumulation by 1 μM CPA was investigated in cells pretreated with various concentrations of CCPA (0.1 nM - 1 μM) for 16 hr (data not shown). Cells incubated with 0.1 μM CCPA for 16 hr at 37°C followed by four wash cycles showed a 48% reduction in specific [^{3}H]CPX binding (control, 0.4 pmol/mg protein) with no change in the K_D value for the

remaining receptors labeled with [^{3}H]CPX (control, 0.5 nM; CCPA-pretreated, 0.4 nM).

The concentration-response relationships of CPA, NECA and WRC-0018 induced attenuation on ISO-stimulated cAMP accumulation in control and CCPA-pretreated cells are shown in Figures 3-20, 3-21 and 3-22, respectively. In control cells, CPA decreased cAMP content in a concentration-dependent manner with an EC_{50} value of 1.2 nM and maximal inhibition of 78% (Figure 3-20). In comparison, after pretreatment of the cells with CCPA, the EC_{50} value for CPA-induced inhibition on ISO-stimulated cAMP accumulation was increased by 13-fold (EC_{50}, 15 nM) without any significant change in the maximal inhibition. Pretreatment of the cells with 0.1 μM CCPA for a longer period (68 hr) did not cause a further shift to the right of the concentration response curve of CPA and had no effect on the maximal response of this agonist (data not shown). As illustrated in Figure 3-21, in control cells, NECA decreased cAMP content in a concentration-dependent manner with an EC_{50} value of 7.2 nM and maximal inhibition of 75%. After pretreatment of the cells with CCPA, the EC_{50} value for NECA-induced inhibition on ISO-stimulated cAMP accumulation was increased by 17-fold (EC_{50}, 120 nM) without any significant change in the maximal inhibition. The effect of pretreatment of the cells with CCPA on WRC-0018 induced attenuation on ISO-stimulated cAMP accumulation is depicted in Figure 3-22. In control cells, WRC-0018 decreased the cAMP content with an EC_{50} value of 830

nM and caused a maximal inhibition of 77%. After pretreatment of the cells with CCPA, the inhibition of ISO-stimulated cAMP accumulation by WRC-0018 was markedly attenuated with a maximal inhibition of cAMP accumulation of about 20%.

The effects of pretreatment of cells with CCPA on the A_2-AdoR mediated effect of WRC-0018 is shown in Figure 3-23. The control data from Figure 3-4 were replotted in Figure 3-23. In CCPA-pretreated cells, the WRC-0018 mediated attenuation of cAMP accumulation was abolished. The EC_{50} of the WRC-0018 mediated stimulation of cAMP accumulation was 17 nM. The maximal stimulation of cAMP accumulation was not significanly increased (control, 8±1 -fold above basal; CCPA-pretreated, 3±0 -fold above basal). The effects of pretreatment of the cells with CCPA on the A_2-AdoR mediated response of NECA is shown in Figure 3-24. The data for NECA in control cells were replotted from Figure 3-13. In CCPA-pretreated cells, NECA did not stimulate cAMP accumulation, however, the basal level of cAMP was significantly increased ($p \leq 0.0005$) from 3±2 in control cells to 11±1 pmol/mg protein/min in pretreated cells. A longer time of preincubation of the cells with CCPA (68 hr) also failed to unmask a NECA mediated A_2-AdoR mediated increase in cAMP accumulation (data not shown). The effects of pretreatment of the cells with CCPA on the A_2-AdoR mediated response of ADO is shown in Figure 3-25. The data for ADO in control cells were reploted from Figure 3-14. In CCPA-pretreated cells, ADO did not stimulate cAMP accumulation.

In summary, these data show that after CCPA-pretreatment, the selective A_2-AdoR agonist WRC-0018 causes a sustained A_2-AdoR mediated stimulatory effect on cAMP accumulation. However, the A_2-AdoR mediated stimulatory effects of the nonselective agonists NECA and ADO were still not uncovered after pretreatment of DDT cells with CCPA.

Effect of Adenosine on the Desensitization of A_1- and A_2-AdoR Systems

The endogenous agonist for the AdoR is ADO which has been shown to be a nonselective agonist (Olsson and Pearson, 1990; Londos et al., 1980). In a separate series of experiments, I investigated whether ADO had differential effects on the desensitization of AdoR subtypes and hence, determined if the expression of the A_2-AdoR mediated response could be uncovered. Cells were pretreated with 100 μM ADO to ensure that both receptor subtypes were stimulated with the agonist.

Desensitization of A_1-AdoR

The effect of ADO on the desensitization of A_1-AdoR was investigated by studying the effect of CPA to inhibit ISO-stimulated cAMP accumulation in control (untreated) and ADO (100 μM, 24 hr)-pretreated cells (Figure 3-26). DIP and EHNA were present during the period of pretreatment of the cells with ADO to prevent ADO metabolism and thereby maintain ADO concentration in the incubation medium relatively constant. As depicted in Figure 3-26, in control cells, CPA decreased

cAMP content in a concentration-dependent manner with an EC_{50} value of 2.0 nM and maximal inhibition of 77%. In comparison, after pretreatment of the cells with ADO, the EC_{50} value for CPA-induced inhibition on ISO-stimulated cAMP accumulation was increased by 15-fold (EC_{50}, 30 nM) without any significant change in the maximal inhibition (pretreated, 73%).

Desensitization of A_2-AdoR

The effect of ADO on the desensitization of A_2-AdoR were investigated by studying the effect of WRC-0018 on cAMP accumulation (Figure 3-27). In control and DIP+EHNA-pretreated cells, WRC-0018 produced a biphasic concentration response curve. At low concentrations (1 - 500 nM), WRC-0018 stimulated cAMP accumulation with a maximal increase of 4-fold above the basal level. At higher concentrations (>500 nM) of WRC-0018, the cAMP accumulation was attenuated. After pretreatment of the cells with ADO, the basal level of cAMP was significantly increased ($p \leq 0.025$) from 3±1 to 9±1 pmol cAMP/mg protein/min. However, WRC-0018 did not stimulate cAMP accumulation above the basal level.

The effect of ADO on cAMP accumulation after pretreatment of the cells with ADO is shown in Figure 3-28. In both control or ADO-pretreated cells, ADO did not stimulate cAMP accumulation above the basal level.

Discussion

The interaction of the inhibitory A_1-AdoR and the stimulatory A_2-AdoR was investigated using DDT cells. The co-expression of both AdoR subtypes was first shown in this cell line by Ramkumar et al. (1990). In addition, ßARs which mediate the stimulation of cAMP accumulation are also expressed in DDT cells (Norris et al., 1983).

In the presence of a constant concentration of ISO which increased cAMP accumulation, several A_1-AdoR agonists inhibited the ISO stimulatory effect in a concentration-dependent manner. Furthermore, in the presence of a fixed concentration of A_1-AdoR agonists, the effect of ISO on cAMP accumulation was greatly attenuated. The inhibitory effect of CPA on ISO-stimulated cAMP formation was blocked by 1 µM CPX, a selective A_1-AdoR antagonist (data not shown). These findings indicate that CPA exerts its inhibitory effect on ßAR mediated cAMP accumulation by activating A_1-AdoRs which are known to be negatively coupled to AC. This A_1-AdoR mediated inhibition on cAMP formation confirms and extends the recent reports on AdoRs in DDT cells (Ramkumar et al., 1990; Gerwins et al., 1990; Gerwins and Fredholm, 1991). One difference between the present data and the previous reports is that NECA produced a 97% maximal inhibition of cAMP accumulation in intact DDT cells in our experiments. However, only a 30% maximal inhibition of AC activity in DDT cell membranes by NECA was reported by Ramkumar et al.(1990). The

lower maximal inhibition of AC activity in DDT cell membranes may be due to the homogenization process during membrane preparation affecting the function of A_1-AdoR.

Our experiments also suggest that the stimulation of cAMP by low concentrations of WRC-0018 was mediated by an A_2-AdoR. Evidence supporting this include the inhibitory effect of the AdoR antagonist 8PST and lack of effect of the selective A_1-AdoR antagonist N-0861 on the stimulatory response of WRC-0018. Since a selective A_2-AdoR antagonist has yet to be synthesized, the next best candidate, 8PST, which is only slightly more selective to A_1-AdoR with a $K_i(A_2)/K_i(A_1)$ ratio of 5.9 (Trivedi et al., 1990) was used.

Interestingly, low concentrations of WRC-0018 which increase cAMP accumulation failed to potentiate ISO-stimulated cAMP accumulation (Figure 3-7). This may be because A_2-AdoR and ßAR share the same pool of AC for cAMP production and the system may be maximally stimulated at 10 µM ISO. Also observed was a greater stimulatory effect of ISO on cAMP accumulation (3 times greater) as compared with WRC-0018. This finding could be explained by 1) a higher density of ßAR on DDT cells, 2) a higher coupling efficiency of ßAR for the G_s protein or 3) an easier access of ßAR to the AC pool.

The A_1-AdoR agonist CPA inhibited the A_2-AdoR stimulatory effect of WRC-0018 in our experiment suggesting that the inhibitory A_1-AdoRs dominate over the stimulatory A_2-AdoRs in DDT cells. This dominant role of A_1-AdoR may also

explain two observations. First, the assumed nonselective agonists NECA and ADO showed no stimulation of cAMP accumulation. At effective concentrations of either agonist, both AdoR subtypes would be activated, but the A_1-AdoR mediated inhibition would predominate and hence inhibit the A_2-AdoR mediated stimulation of AC and thereby accumulation of cAMP. However, this finding is in contrast to the observation reported by Ramkumar et al. (1990). NECA (10 μM), in absence of any A_1-AdoR antagonist, produced a 2.1 fold stimulation of AC activity over the basal level in DDT cell membranes (Ramkumar et al., 1990). The discrepancy between this report and our data may be due to the homogenization process during membrane preparation affecting the function of A_1-AdoR. The second observation which could be explained by the dominant role of A_1-AdoR is the downward portion of the WRC-0018 biphasic concentration response may reflect the activation of the A_1-AdoR. This was indicated by the inhibitory effect of WRC-0018 on ISO-stimulated cAMP accumulation over the same concentration range where the downward part of the biphasic response of WRC-0018 occurred (Figure 3-7).

Three approaches were used to study the expression of A_2-AdoR mediated responses and further establish that the downward phase of the WRC biphasic response is A_1-AdoR mediated. The first approach involved the use of the selective A_1-AdoR antagonist N-0861. The selectivity of N-0861 for the A_1-AdoR was tested by receptor binding and cAMP

accumulation. Several lines of evidence support the high selectivity of N-0861 for A_1-AdoR. These include 1) a concentration dependent displacement of specific [^{3}H]CPX binding at the A_1-AdoR site by N-0861 with a Hill slope of 1, indicating the interaction of this antagonist with a single class of binding sites, 2) the substantial attenuation of the CPA mediated inhibition of cAMP accumulation by 10 μM N-0861, and 3) the unaltered stimulatory effect of WRC-0018 by N-0861 at concentrations ranging from 0.1 nM - 10 μM.

In the presence of N-0861, the stimulatory response of WRC-0018 was sustained, i.e., the downward portion of the biphasic response was abolished. This finding is consistent with the hypothesis that the downward portion of the WRC-0018 biphasic response was A_1-AdoR mediated. Assuming that the interaction of N-0861 with the A_1-AdoR and the interaction of WRC-0018 with A_1- and A_2-AdoR are reversible and competitive, then in the presence of N-0861, the downward portion of WRC-0018 concentration response should be shifted to the right. This was not tested in the present study due to the insolubility of WRC-0018 to obtain and test concentrations above 100 μM. Similar to the results with WRC-0018, blockade of the A_1-AdoR with N-0861 uncovered a cAMP stimulation response of the agonists NECA (≤100 μM) and ADO (≤5 μM). These results are in keeping with the observation on NECA mediated A_2-AdoR stimulatory response on AC activity reported by Ramkumar et al. (1990). However, at ≥10 μM NECA (Figure 3-13) and 10 μM ADO (Figure 3-14), there was a decrease in cAMP

accumulation. This attenuation may be due to the higher concentrations of NECA and ADO overcoming the N-0861 (10 μM) blockade of the A_1-AdoR allowing its activation.

The second approach used to investigate the expression of A_2-AdoR mediated response was uncoupling of the A_1-AdoR inhibitory effect with PTX. It is well established that PTX selectively ADP-ribosylates the α_i subunit. This covalent modification inactivates the G_i protein and uncouples inhibitory receptors including the A_1-AdoR resulting in loss of receptor mediated inhibition of AC activity (Gilman, 1987; Nathanson, 1987; Hazeki and Ui, 1981). Experiments showed that the inhibition of AC activity by (R)-PIA in DDT cells was greatly attenuated after pretreatment of the cells with 100 ng/ml PTX for 18 hrs (Ramkumar et al., 1990). In the present study, the ability of CPA to inhibit ISO-stimulated cAMP accumulation was used as means to determine the effect of PTX-pretreatment on the A_1-AdoR mediated inhibition of cAMP accumulation. PTX (25 ng/ml)-pretreatment for 18 hr was found to be sufficient to cause complete loss of the CPA inhibitory effect. In PTX-pretreated cells, the downward phase of the WRC-0018 biphasic response was completely eliminated. Furthermore, NECA and ADO which had no effect on cAMP accumulation in untreated cells stimulated cAMP accumulation in cells pretreated with PTX. The result with NECA, i.e., the stimulation of cAMP accumulation in PTX-pretreated cells, is also consistent with other reports (Gerwins et al., 1990). That is, NECA (10 μM) was reported to

increase cAMP content 1.5 fold above the basal level in DDT cells pretreated with 200 ng/ml PTX for 4 hr (Gerwins et al., 1990). Our data strongly suggest that the downward phase for WRC-0018 concentration response is due to activation of an inhibitory receptor. The EC_{50} values of the A_2-AdoR effect of WRC-0018 in the presence of N-0861 (93 nM) and after PTX-pretreatment (90 nM) were very similar. In addition, similarity also exists between the maximal responses of the A_2-AdoR effect of WRC-0018 in the presence of N-0861 (15±6 -fold above basal) and after PTX-pretreatment (14±3 -fold above basal). These results suggest that N-0861 and PTX have a similar net blocking effect on the A_1-AdoR mediated inhibitory action of WRC-0018 and are unlikely to affect the interaction of WRC-0018 with the A_2-AdoR. As a consequence, the A_2-AdoR mediated stimulatory action of WRC-0018 is sustained to the same extent.

Selective desensitization and/or down-regulation of A_1-AdoR was the third approach used. Chronic pretreatment (16 hr) of cells with the selective A_1-AdoR agonist CCPA (0.1 μM) caused a 48% loss of A_1-AdoR. This indicates that the A_1-AdoR is down-regulated after chronic pretreatment with a selective A_1-AdoR agonist. Down-regulation of receptors after long term exposure to an agonist is a widely reported phenomenon. However, the loss of A_1-AdoR during down-regulation is significantly less in comparison with other receptor systems. For instance, after 1321N1 astrocytoma cells were incubated with ISO for 12-24 hr, greater than 90% of the ßARs were lost

from the cells (Doss et al., 1981). In DDT cells, down-regulation of ßAR occurred rapidly with a $t_{1/2}$ of about 3 hr and proceeded to 80-85% loss of receptors by 13 hr of incubation of the cells with 10 µM epinephrine (Toews, 1987).

Desensitization of A_1-AdoR system was studied by examining the concentration responses of CPA, NECA and WRC-0018 to inhibit ISO-stimulated cAMP accumulation in control and CCPA-pretreated cells. For CPA (Figure 3-20) and NECA (Figure 3-21), the concentration response curves were shifted to the right (10-20 -fold) without any change in the maximal responses. Longer pretreatment periods did not increase the magnitude of shift (data not shown) indicating that maximal desensitization was achieved. The shift in the concentration response is not surprising because the sensitivity of the cells to the agonist should decrease as the receptor number decreases provided little or no change in the affinity of the agonist for the remaining receptors. The ability of CPA and NECA to produce the same maximal response in control (untreated) cells and CCPA-pretreated cells where the receptor number is decreased by almost 50% may be explained by the presence of spare A_1-AdoRs. In this situation, the responsiveness is not directly proportional to receptor occupancy and the maximal response can be obtained when less than 100% of the receptors are activated. Many studies have shown the existence of spare receptors in other systems (Stephenson, 1956; Nickerson, 1956; Nelson et al., 1986; Gunst et al., 1989). For example, in guinea pig lung, after

≈50% of ßARs were inactivated by an irreversible antagonist, the maximal airway responsiveness to ISO was still maintained (Nelson et al., 1986). In canine trachealis muscle, the maximal contractile response for acetylcholine was achieved when only 4% of muscarinic receptors were occupied (Gunst et al., 1989).

Interestingly, in control cells, WRC-0018 produced the same maximal inhibitory response on ISO-stimulated cAMP accumulation as CPA. However in CCPA-pretreated cells, the maximal response produced by WRC-0018 was greatly reduced (Figure 3-22) whereas the maximal response achieved by CPA was not changed from that in control cells. This indicated that WRC-0018 acted as a full A_1-AdoR agonist (as compared with CPA) in control cells whereas it acted as a partial agonist in pretreated cells. One explanation for this may be related to the intrinsic efficacy of the agonists. In control cells, WRC-0018 may need to occupy more receptors than CPA to achieve its maximal response. Thus, for WRC-0018, there may be little or no spare A_1-AdoRs and the responsiveness may be more directly related to receptor occupancy. As the receptor number decreases, the maximal response for WRC-0018 will be reduced as was observed after chronic CCPA-pretreatment. A desensitization-induced change in agonist efficacy has also been reported for some ßAR agonists. In membranes prepared from untreated L6 skeletal muscle cells, several compounds acted as full ßAR agonists when compared with ISO, but after desensitization of the ßAR system, these compounds acted as

partial agonists (Pittman et al., 1984). Alternative explanations may also be possible. For example, there may be subpopulations of A_1-AdoRs in DDT cells where CPA is nonselective and WRC-0018 is selective for only one subtype which was selectively down-regulated and desensitized by chronic CCPA-pretreatment. Further experiments will be necessary to determine the reason for the differential changes between CPA and WRC-0018 responsiveness after a decrease in receptor number.

Because CCPA-pretreatment had little effect on the A_2-AdoR mediated stimulation component of the WRC-0018 concentration response (Figure 3-23), this suggests that CCPA has little or no desensitization effect on the A_2-AdoR. On the other hand, chronic CCPA-pretreatment abolished the downward phase of the WRC-0018 biphasic concentration response curve (Figure 3-23). CCPA is a highly selective A_1-AdoR agonist (Lohse et al., 1988). The loss of the downward phase of the WRC-0018 response in conjunction with a desensitization and/or down-regulation of A_1-AdoR is consistent with the downward phase being A_1-AdoR mediated. Thus, the elimination of the downward phase may be due to 1) desensitization and/or down-regulation decreasing the sensitivity to A_1-AdoR agonists thereby further increasing the A_2-AdoR selectivity of WRC-0018, and 2) the loss of efficacy of WRC-0018 as an A_1-AdoR agonist in the partially desensitized state.

In contrast to the effects of N-0861 and PTX, chronic CCPA-pretreatment did not uncover any NECA or ADO stimulation of cAMP accumulation. This observation can be explained by the differences in the agonist's sensitivity to produce inhibition or stimulation of cAMP accumulation.

Summary of EC_{50} Values (nM)

Agonists	A_1-AdoR		A_2-AdoR
	Control	CCPA	(in PTX-pretreated cells)
WRC-0018	830	-	90
NECA	7.2	120	180
ADO	97	-	>1000
CPA	1.2	15	-

In the case of NECA, the estimated EC_{50} values for the inhibition (A_1-AdoR effect) and stimulation (A_2-AdoR effect) of cAMP accumulation in untreated cells were 7.2 and 180 nM, respectively. The latter estimate was derived from the NECA concentration response curve after PTX-pretreatment and this value was based on the assumption that PTX-pretreatment had little or no effect on the potency of this agonist to stimulate cAMP accumulation. The higher potency of NECA to inhibit cAMP accumulation indicates that this agonist shows some degree of selectivity for the A_1-AdoR over the A_2-AdoR in DDT cells. In contrast, NECA has been reported to be a nonselective agonist in other systems (Hutchinson et al, 1990). After chronic CCPA-pretreatment to partially down-

regulate the A_1-AdoR, the EC_{50} value for the NECA inhibitory response was increased from 7.2 nM in control cells to 120 nM in CCPA-pretreated cells which was similar to the EC_{50} value for stimulation (180 nM) (see the table above). That is, the EC_{50} value for NECA-induced cAMP accumulation (A_2-AdoR effect) is still greater than NECA's EC_{50} value for inhibition of cAMP accumulation (A_1-AdoR effect). Similar EC_{50} values for NECA to inhibit and stimulate cAMP accumulation in cells chronically pretreated with CCPA indicates that this agonist would occupy both receptor subtypes, but activation of the A_1-AdoR would prevent expression of an A_2-AdoR response. It appears that the expression of an A_2-AdoR response in untreated cells requires a selective A_2-AdoR agonist. This was evident with WRC-0018 which based upon its stimulatory and inhibitory concentration ranges, showed an approximately 500-600 -fold selectivity for the A_2-AdoR (Figure 3-4). Although chronic CCPA-pretreatment reduced the sensitivity for the A_1-AdoR response of NECA, this reduction was not sufficient to result in activation of the A_2-AdoR without significant stimulation of the A_1-AdoR. A similar explanation may account for the lack of an ADO mediated stimulation of cAMP accumulation in cells chronically pretreated with CCPA. The EC_{50} for ADO to inhibit and stimulate (in PTX-pretreated cells) cAMP accumulation was 97 nM and >1 μM, respectively. This A_1-AdoR selectivity for ADO is in contrast to previous studies in other cell types where this agonist has been shown to be nonselective (Londos et al., 1980; Olsson and Pearson, 1990).

Assuming a 10-20 fold reduction in ADO sensitivity (like CPA) to inhibit cAMP accumulation in cells chronically pretreated with CCPA, this would not be sufficient to allow selective expression of the A_2-AdoR response. However, it will be of interest to determine if selective down-regulation of the A_1-AdoR would reduce the sensitivity of a non-selective agonist sufficient to uncover expression of the A_2-AdoR response.

Interestingly, CCPA-pretreatment was found to increase the basal cAMP level (Figure 3-24). Desensitization of inhibitory receptors resulting in an increase in basal cAMP levels or potentiation of stimulatory receptor effects has been widely reported. For example, a 3-fold increase in basal cAMP level was observed after pretreatment of NG108-15 cells with 10 μM carbachol for 19 hr (Nathanson et al., 1978). This increase in basal cAMP level may be due to the loss of an inhibitory tone on AC activity mediated by the G_i protein. The inhibitory tone may involve the basal dissociation of G_i (in the absence of an inhibitory agonist) with the α_i subunit directly attenuating the activity of the catalytic subunit of AC (Gilman, 1987). Several mechanisms may be responsible for the loss of the inhibitory tone. First, chronic CCPA-pretreatment may decrease the cellular content of G_i protein. Evidence supporting this contention has been reported by Green (1987) who showed a decrease in G_i protein after desensitization of the A_1-AdoR system in primary culture of rat adipocytes. Second, CCPA-pretreatment may induce an impairment in the function of G_i. For example, a CCPA-induced

phosphorylation of G_i may prevent the basal release of the activated α_i subunit.

Because A_1-AdoR inhibition of cAMP accumulation dominated over A_2-AdoR mediated stimulation, it was of interest to investigate whether the endogenous agonist ADO would have a differential desensitization effect on the receptor subtypes when both were chronically stimulated. After pretreatment of cells with 100 μM ADO for 24 hr, the concentration response of CPA to inhibit ISO-stimulated cAMP accumulation shifted to the right indicating that the A_1-AdoR system was desensitized (Figure 3-26). This was similar to that observed with CCPA-pretreatment. However, WRC-0018 did not increase cAMP accumulation in ADO-pretreated cells, suggesting that the A_2-AdoR system was also desensitized (Figure 3-27). In keeping with this conclusion, ADO also did not stimulate cAMP accumulation in ADO-pretreated cells. The observation that the A_1-AdoR was still present (albeit at reduced agonist sensitivity) whereas the A_2-AdoR response was abolished indicates that the extent of desensitization was different for each receptor subtype. This may be explained by one or more factors: 1) the density of A_1-AdoR in DDT cells was higher than that of A_2-AdoR. An A_1-AdoR to A_2-AdoR ratio of 4:1 in DDT cells has been reported by Ramkumar et al. (1990), 2) the coupling efficiency between the receptors and their second messenger system may be different, 3) the rate of desensitization of A_2-AdoR was much faster than that of A_1-AdoR. Ramkumar et al. (1991) reported that the $t_{1/2}$ for the

desensitization of A_1- and A_2-AdoR was 8 and 0.75 hr in DDT cells, respectively, 4) the efficacy of the agonist to induce desensitization of the receptor subtypes may be different, or 5) the mechanism for desensitization of the receptor subtypes may be different. Evidence for a differential desensitization mechanism has been reported recently (Ramkumar et al., 1991). During desensitization, the A_1-AdoR was down-regulated (internalized), uncoupled from G proteins and phosphorylated whereas the A_2-AdoR was not (Ramkumar et al., 1991).

Summary

Adenosine is an autocoid produced by the same cells on which and/or adjacent cells it exerts its effects. The results of the present study demonstrate that in DDT cells adenosine acts on at least two receptor subtypes (A_1- and A_2-AdoR) whose actions result in opposing effects on the formation of cAMP. There may be circumstances in which adenosine production rapidly and transiently increases and thereby maximally activates AdoRs. When the concentration of adenosine rises far above the physiological range, the coexistence of two receptor subtypes on the same cell with opposing functional effects would dampen the responses to the transient extremes of adenosine concentration. That is, in the example illustrated in Figure 3-14 (ADO alone), activation of the A_1-AdoR would attenuate the effect of A_2-

AdoR activation of AC and thereby dampen a rise in cellular cAMP.

The data from the present study show that in DDT cells activation of the inhibitory A_1-AdoR will predominate and thus mask the stimulatory A_2-AdoR response. This observation may have broader implications because other cell types and tissues have been reported to express both A_1- and A_2-AdoRs. These include porcine coronary vascular smooth muscle cells (Mills and Gewirtz, 1990), FRTL-5 cells derived from normal rat thyroid (Nazarea et al., 1991), ventricular myocytes from chick embryo (Xu et al., 1992) and heart tissue (Olsson and Pearson, 1990).

Our data demonstrate that cells with both receptor subtypes can be pharmacologically manipulated to uncover an A_2-AdoR response with a highly selective A_2-AdoR agonist or with the use of a selective A_1-AdoR antagonist. There may be situations where these pharmacological approaches have therapeutic potential and suggest possible strategies for drug development. For example, in cells or tissues where both receptor subtypes are expressed, the A_2-AdoR mediated responses may be suppressed under normal conditions. However, by selectively blocking the A_1-AdoR system or activating the A_2-AdoR with a highly selective agonist, A_2-AdoR mediated responses (e.g., vasodilation to increase blood flow, inhibition of platelet aggregation during thrombosis, generation of superoxide radicals to prevent reperfusion injury) may be expressed for possible therapeutic effect.

Another implication of our study with potential therapeutic value is the possibility to decrease the side effects caused by nonselective AdoR agonists. For instance, adenosine and its analogs may activate both receptor subtypes and hence, if one subtype can be selectively blocked, the side effects mediated by this receptor subtype should be attenuated.

The development of more selective agonists and antagonists may be needed to increase the concentration range over which a response can be achieved. Thus, the ultimate objective would be to develop specific agonists and antagonists.

Finally, in cell types in which the A_1- and A_2-AdoR coexist, the question arises as to whether an A_2-AdoR mediated response initiated by ADO would ever be expressed under physiological or pathological conditions? Although A_1-AdoR suppression of the A_2-AdoR response may be normal under most physiological conditions (at least in cells that express both receptor subtypes), the A_2-AdoR response may be needed under some pathological conditions. For example, under stress, the chronic release of adenosine or other cellular mechanisms may result in the loss of the A_1-AdoR or other components of its signal transduction pathway (e.g., G_i protein) allowing A_2-AdoR expression. Although our data indicated that chronic stimulation of A_1- and A_2-AdoR resulted in desensitization of both receptors and Ramkumar et al. (1991) found that the rate of desensitization for the A_2-AdoR

was faster than that for the A_1-AdoR in DDT cells, in other cells, the opposite may occur. Thus, additional studies are necessary to test this latter possibility, further characterize the dual modulation of AC by adenosine in the same cell and/or organ and define the implications for such role.

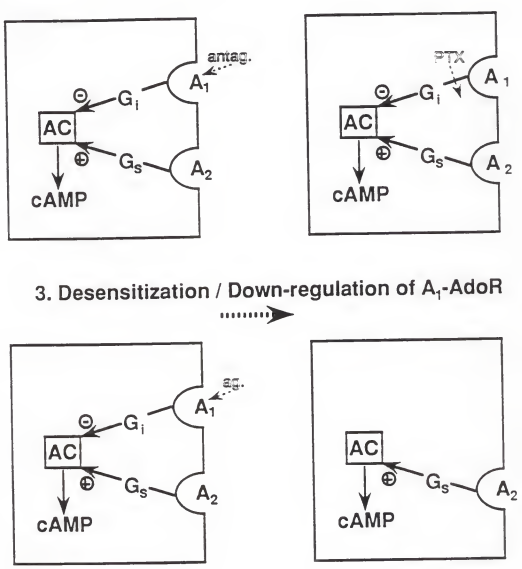

Figure 3-1. Experimental approaches to express the A_2-AdoR mediated response.

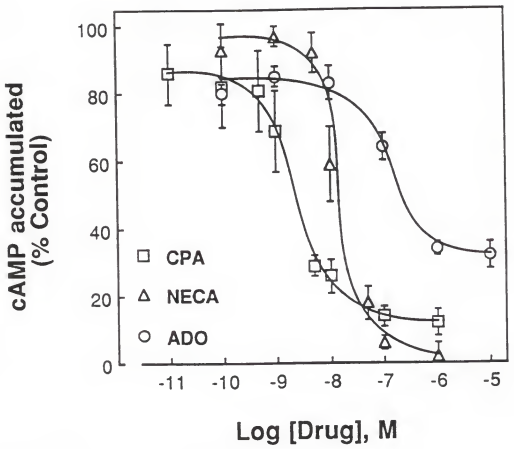

Figure 3-2. Inhibition of ISO-stimulated cAMP accumulation in DDT cells by CPA, NECA and ADO. Cells were incubated in HBSS containing 50 μM rolipram, 10 μM ISO or ISO plus the indicated concentrations of CPA, NECA or ADO + 1 μM DIP + 1 μM EHNA for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The control cAMP accumulated in the presence of ISO alone was 57±12, 87±9 and 68±4 pmol/mg protein/min for the CPA, NECA and ADO experiments, respectively. The basal level of cAMP accumulated was below the detection level for CPA and NECA and 6±1 pmol/mg protein/min for the ADO experiment. Each data point is the mean±SD of quadruplicate determinations and is representative of 2 experiments.

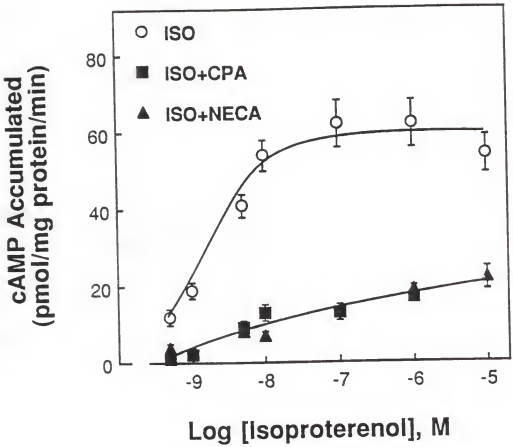

Figure 3-3. The effect of CPA and NECA on ISO-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 µM rolipram, the indicated concentrations of ISO, without or with 0.1 µM CPA or 1 µM NECA for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The basal level of cAMP accumulated was 1±1 pmol/mg protein/min. Each data point is the mean±SE, n=3 - 5.

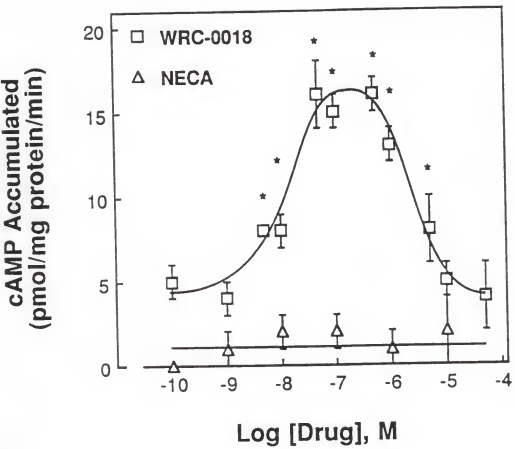

Figure 3-4. The effect of WRC-0018 and NECA on cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 μM rolipram and the indicated concentrations of WRC-0018 or NECA for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The basal level of cAMP accumulated was 3±1, 3±2 pmol/mg protein/min and each data point is the mean±SE, n=15 for WRC-0018 and n=3 for NECA. The * indicates a $p<0.01$ for each WRC-0018 data point as compared to its basal level.

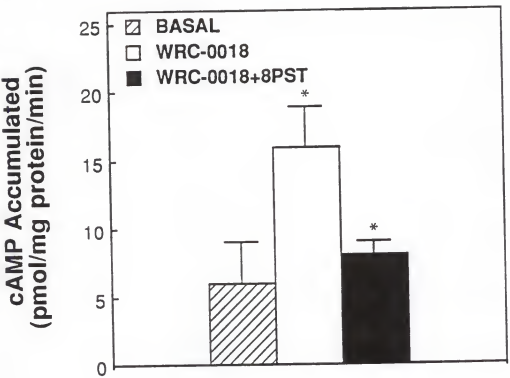

Figure 3-5. The effect of 8PST on WRC-0018-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 μM rolipram, without or with 0.1 μM WRC-0018 or WRC-0018 plus 5 μM 8PST for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SD of quadruplicate determination. The * indicates a $p<0.01$ for the WRC-0018 value compared to the basal value and a $p<0.005$ for the WRC-0018 plus 8PST value compared to the WRC-0018 value.

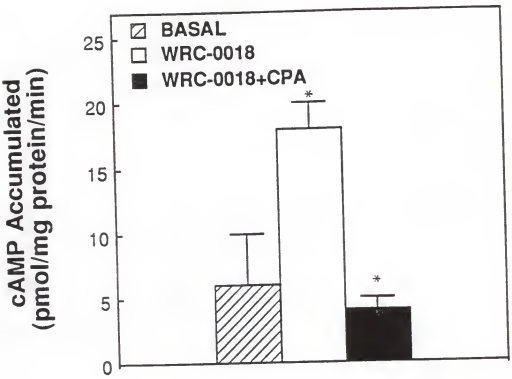

Figure 3-6. The effect of CPA on WRC-0018-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 µM rolipram, without or with 0.1 µM WRC-0018 or WRC-0018 plus 1 µM CPA for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SD of quadruplicate determination. The * indicates a $p<0.005$ for the WRC-0018 value compared to the basal value and for the WRC-0018 plus CPA value compared to the WRC-0018 value.

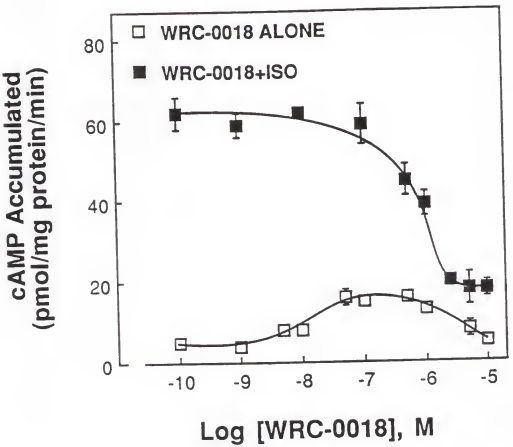

Figure 3-7. Effect of WRC-0018 on ISO-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 µM rolipram, the indicated concentrations of WRC-0018, without or with 10 µM ISO for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The control cAMP accumulated in the presence of ISO alone was 62±9 pmol/mg protein/min. Each data point is the mean±SE, n=4.

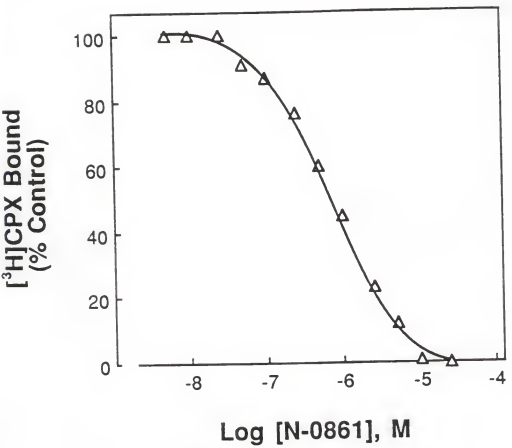

Figure 3-8. Inhibition of specific [^{3}H]CPX binding to DDT cell membrane by N-0861. Membranes protein (320 µg) was incubated with 1 nM [^{3}H]CPX and the indicated concentrations of N-0861 for 150 min at room temperature. Specific binding was determined as described in the "Methods" section. Data points are the mean of triplicate determinations which varied by less than 5%. The control specific [^{3}H]CPX binding was 132 fmol/mg protein.

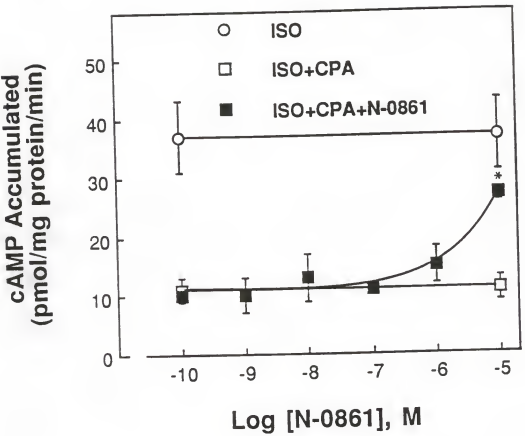

Figure 3-9. The effect of N-0861 on the ability of CPA to inhibit ISO-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 μM rolipram, 10 μM ISO, without or with 0.1 μM CPA or CPA plus the indicated concentrations of N-0861 for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SD of quadruplicate determinations. The * indicates a $p<0.0005$ for the ISO + CPA + N-0861 value compared to the ISO + CPA value.

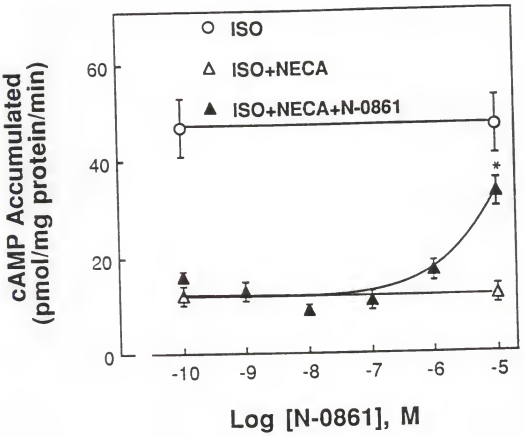

Figure 3-10. The effect of N-0861 on the ability of NECA to inhibit ISO-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 μM rolipram, 10 μM ISO, without or with 1 μM NECA or NECA plus the indicated concentrations of N-0861 for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SD of quadruplicate determination. The * indicates a $p<0.0005$ for the ISO + NECA + N-0861 point from the ISO + NECA point.

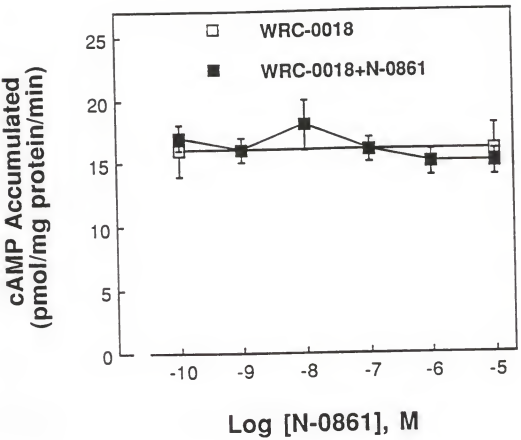

Figure 3-11. The effect of N-0861 on the ability of WRC-0018 to stimulate cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 µM rolipram, 0.1 µM WRC-0018, without or with the indicated concentrations of N-0861 for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The basal level of cAMP accumulated was 6±1 pmol/mg protein/min. Each data point is the mean±SD of quadruplicate determinations.

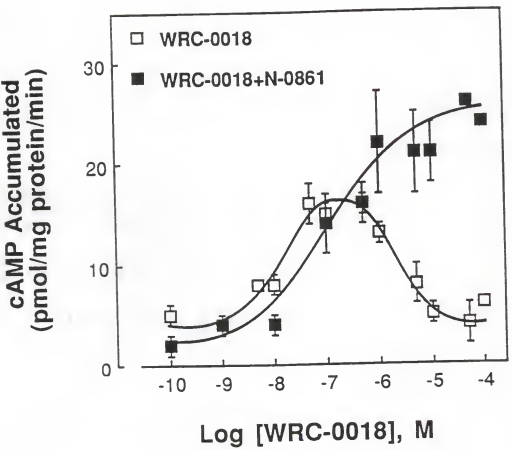

Figure 3-12. The effect of N-0861 on WRC-0018-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 μM rolipram, the indicated concentrations of WRC-0018, without or with 10 μM N-0861 for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The dose response curve of WRC-0018 in the absence of N-0861 is taken from Figure 3-4. The basal level of cAMP accumulated in the presence of N-0861 was 2±1 pmol/mg protein/min. Each data point is the mean±SE, n=3

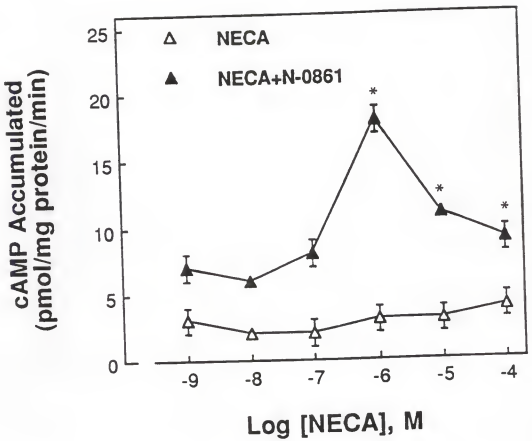

Figure 3-13. The effect of N-0861 on NECA-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 μM rolipram, the indicated concentrations of NECA, without or with 10 μM N-0861 for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The basal level of cAMP accumulated was 1±1 pmol/mg protein/min. Each data point is the mean±SD of quadruplicate determinations and is representative of 2 experiments. The * indicates a $p<0.0005$ for the NECA points from their respective control values.

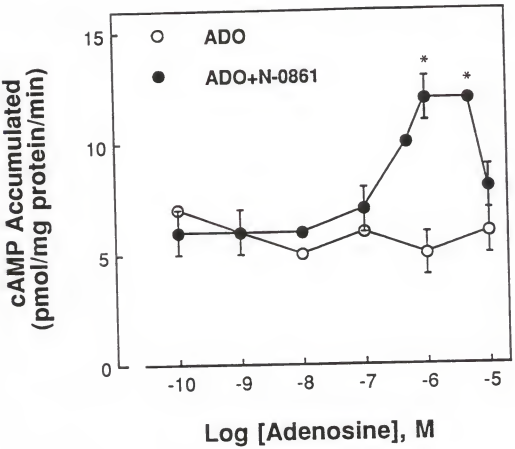

Figure 3-14. The effect of N-0861 on ADO-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 50 µM rolipram, 1 µM DIP, 1 µM EHNA, the indicated concentrations of ADO, without or with 10 µM N-0861 for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The basal level of cAMP accumulated was 6±0 pmol/mg protein/min. Each data point is the mean±SE, n=4. The * indicates a $p<0.01$ for the ADO points from their respective control values.

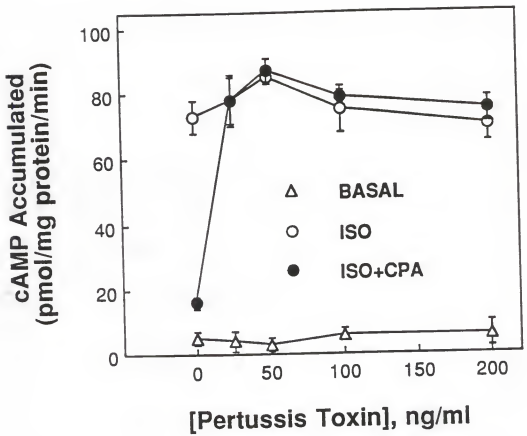

Figure 3-15. The effect of PTX pretreatment on the ability of CPA to inhibit ISO-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media with the indicated concentrations of PTX for 18 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached from the plate. The cells were then incubated in HBSS containing 50 µM rolipram, without or with 10 µM ISO or ISO plus 1 µM CPA for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SD of quadruplicate determination.

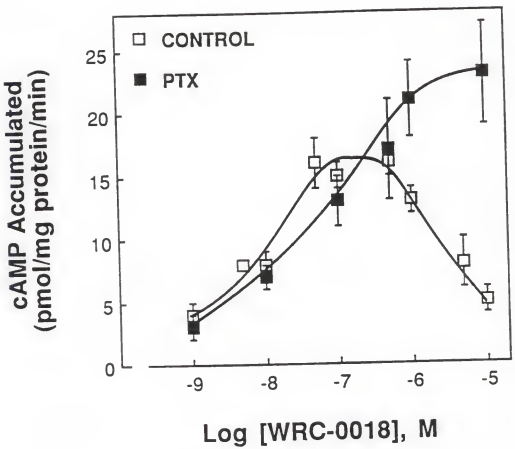

Figure 3-16. The effect of PTX pretreatment on WRC-0018-stimulated cAMP accumulation in DDT cells. Cells were incubated in growth media without or with 25 ng/ml PTX for 18 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. Cells were then incubated in HBSS containing 50 μM rolipram and the indicated concentrations of WRC-0018 for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The dose response curve of WRC-0018 in control cells is taken from Figure 3-4. The basal level of cAMP accumulated was 3±1 and 2±1 pmol/mg protein/min for the control and PTX experiments, respectively. Each data point is the mean±SE, n=6.

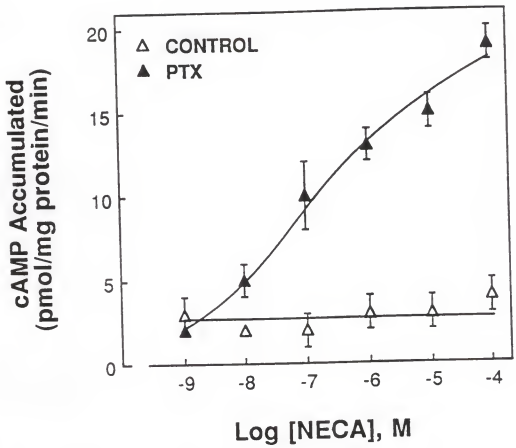

Figure 3-17. The effect of PTX pretreatment on NECA-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media without or with 25 ng/ml PTX for 18 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. Cells were then incubated in HBSS containing 50 μM rolipram and the indicated concentrations of NECA for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The data for NECA in control cells are taken from Figure 3-13. The basal level of cAMP accumulated was 1±1 and 4±2 pmol/mg protein/min for the control and PTX experiments, respectively. Each data point is the mean±SD of quadruplicate determinations and is representative of 2 experiments.

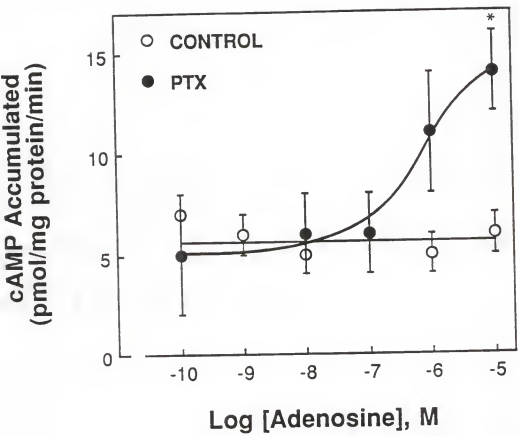

Figure 3-18. The effect of PTX pretreatment on ADO-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media without or with 25 ng/ml PTX for 18 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. Cells were then incubated in HBSS containing 50 µM rolipram, 1 µM DIP, 1 µM EHNA and the indicated concentrations of ADO for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The control data are taken from Figure 3-14. The basal level of cAMP accumulated was 6±0 and 4±1 pmol/mg protein/min for the control and PTX experiments, respectively. Each data point is the mean±SE, n=3. The * indicates a $p<0.05$ for the ADO point from their respective control value.

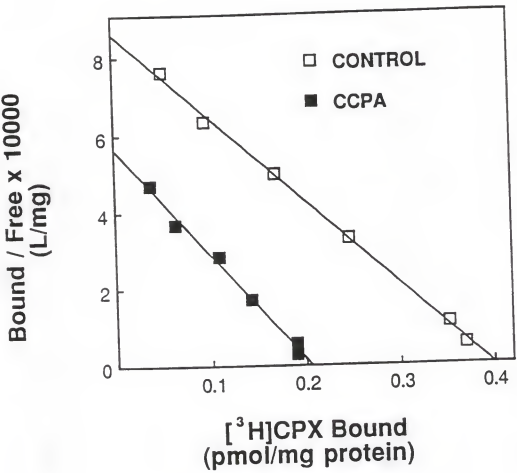

Figure 3-19. Scatchard plot of specific [^{3}H]CPX binding to DDT cell membranes after CCPA treatment. Cells were incubated in fresh growth media without or with 0.1 µM CCPA for 16 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and the membranes prepared. Membrane protein (0.1 mg) was assayed with 0.06 - 4 nM [^{3}H]CPX as described in the "Methods" section. The data are plotted as the ratio of the amount of specifically bound ligand (pmol/mg protein) to free ligand (pmol/L) versus the amount of specifically bound ligand/mg protein. Data points are the mean of triplicate determination and are representative of 3 experiments.

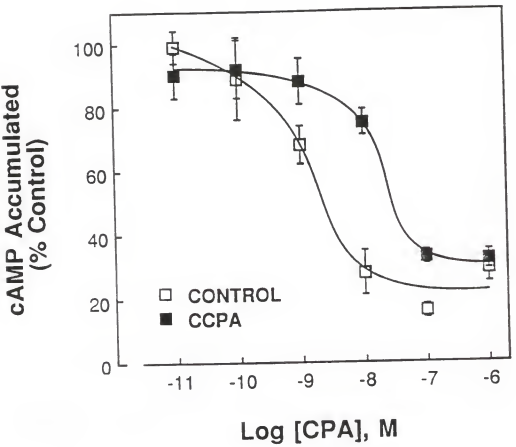

Figure 3-20. The effect of CCPA pretreatment on the ability of CPA to inhibit ISO-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media without or with 0.1 µM CCPA for 16 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. The cells were then incubated in HBSS containing 50 µM rolipram, 10 µM ISO or ISO plus the indicated concentrations of CPA for 7 min at 36°C. The cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SD of quadruplicate determinations and is representative of 2 experiments.

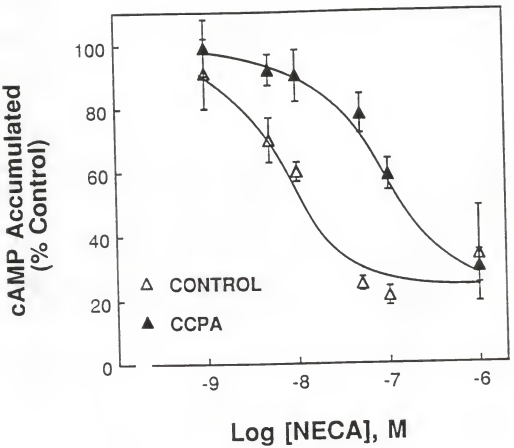

Figure 3-21. The effect of CCPA pretreatment on the ability of NECA to inhibit ISO-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media without or with 0.1 µM CCPA for 16 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. The cells were then incubated in HBSS containing 50 µM rolipram, 10 µM ISO or ISO plus the indicated concentrations of NECA for 7 min at 36°C. The cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SD of quadruplicate determination.

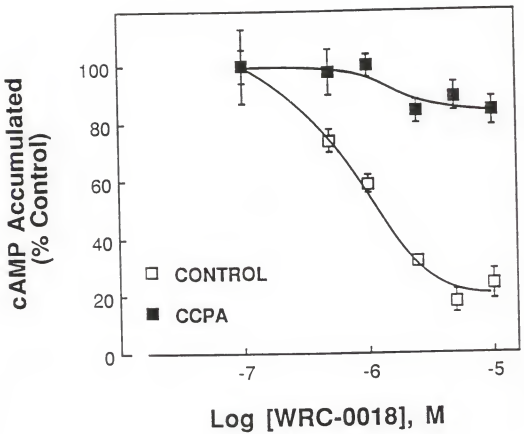

Figure 3-22. The effect of CCPA pretreatment on the ability of WRC-0018 to inhibit ISO-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media without or with 0.1 µM CCPA for 16 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. The cells were then incubated in HBSS containing 50 µM rolipram, 10 µM ISO or ISO plus the indicated concentrations of WRC-0018 for 7 min at 36°C. The cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SD of quadruplicate determinations and is representative of 2-4 experiments.

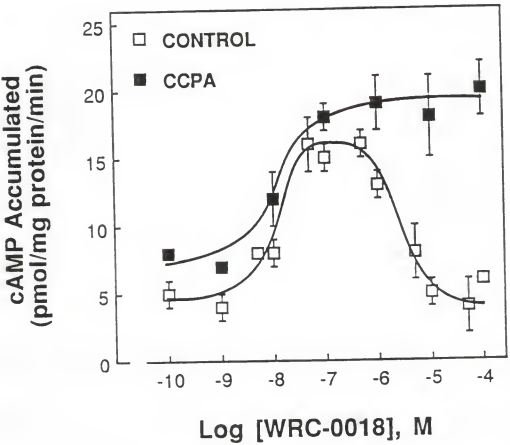

Figure 3-23. The effect of CCPA pretreatment on WRC-0018-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media without or with 0.1 µM CCPA for 16 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. Cells were then incubated in HBSS containing 50 µM rolipram and the indicated concentrations of WRC-0018 for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The dose response curve of WRC-0018 in control cells is taken from Figure 3-4. The basal level of cAMP accumulated was 3±1 and 6±1 pmol/mg protein/min for the control and CCPA experiments, respectively. Each data point is the mean±SE, n=3.

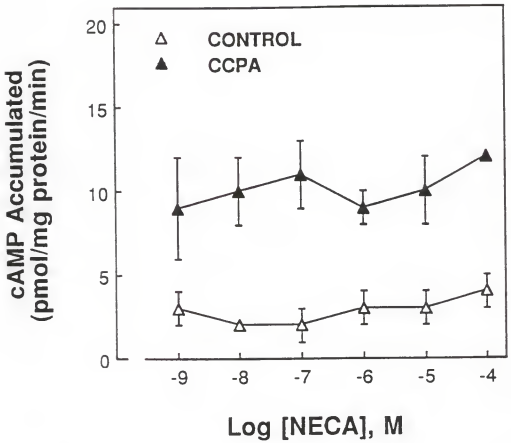

Figure 3-24. The effect of CCPA pretreatment on NECA-stimulated cAMP accumulation in DDT cells. Cells were , incubated in fresh growth media without or with 0.1 µM CCPA for 16 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. Cells were then incubated in HBSS containing 50 µM rolipram and the indicated concentrations of NECA for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The data for NECA in control cells are taken from Figure 3-13. The basal level of cAMP accumulated was 3±2 and 11±1 pmol/mg protein/min for the control and CCPA experiments, respectively. Each data point is the mean±SD of quadruplicate determinations and is representative of 2 experiments.

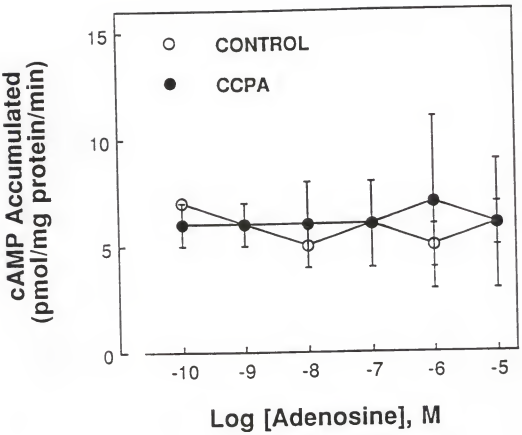

Figure 3-25. The effect of CCPA pretreatment on ADO-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media without or with 0.1 µM CCPA for 16 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. Cells were then incubated in HBSS containing 50 µM rolipram, 1 µM DIP, 1 µM EHNA and the indicated concentrations of ADO for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The control data are taken from Figure 3-14. The basal level of cAMP accumulated was 6±0 and 7±3 pmol/mg protein/min for the control and CCPA experiments, respectively. Each data point is the mean±SE, n=3.

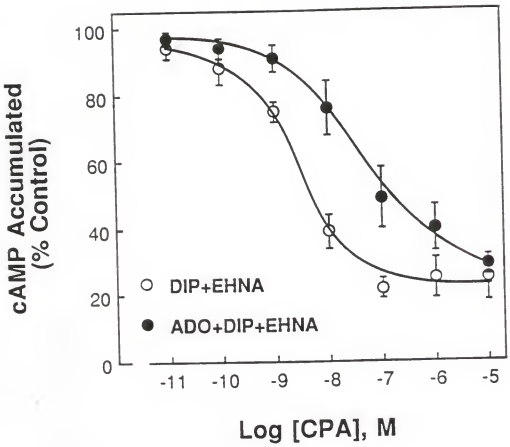

Figure 3-26. The effect of ADO pretreatment on the ability of CPA to inhibit ISO-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media with 5 μM DIP, 1 μM EHNA, without or with 100 μM ADO for 24 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. The cells were then incubated in HBSS containing 50 μM rolipram and 10 μM ISO or ISO plus the indicated concentrations of CPA for 7 min at 36°C. The cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SE, n=5.

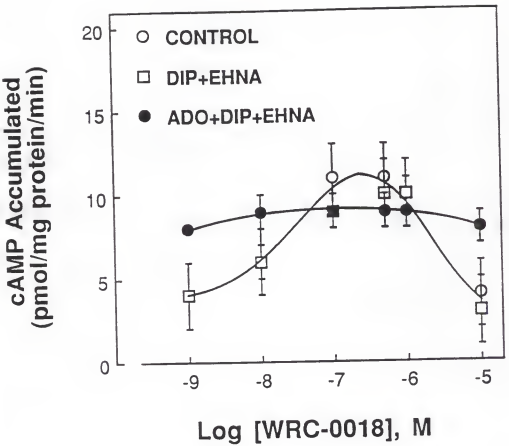

Figure 3-27. The effect of ADO pretreatment on WRC-0018-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media without or with 5 µM DIP + 1 µM EHNA or 5 µM DIP + 1 µM EHNA + 100 µM ADO for 24 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. Cells were then incubated in HBSS containing 50 µM rolipram and the indicated concentrations of WRC-0018 for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The basal level of cAMP accumulated was 3±2, 3±1, 9±1 pmol/mg protein/min for the control, DIP+ENHA and ADO+DIP+EHNA experiments, respectively. Each data point is the mean±SE, n=3.

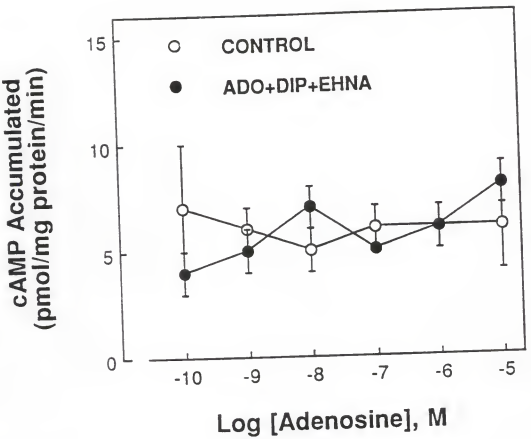

Figure 3-28. The effect of ADO pretreatment on ADO-stimulated cAMP accumulation in DDT cells. Cells were incubated in fresh growth media without or with 5 μM DIP + 1 μM EHNA + 100 μM ADO for 24 hr at 37°C. At the end of the incubation period, the cells were washed 4 times with ice-cold HBSS and detached. Cells were then incubated in HBSS containing 50 μM rolipram, 1 μM DIP, 1 μM EHNA and the indicated concentrations of ADO for 7 min at 36°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The basal level of cAMP accumulated was 6±1, 6±1 pmol/mg protein/min for the control and ADO+DIP+EHNA experiments, respectively. Each data point is the mean±SD of quadruplicate determination.

CHAPTER 4
INHIBITORY EFFECT OF A_1-ADENOSINE RECEPTOR
ON IRREVERSIBLE ACTIVATION OF THE ß-ADRENORECEPTOR

Introduction

The guanine nucleotide binding proteins G_s and G_i play an important role in mediating receptor activation and inhibition of AC activity, respectively. G_s and G_i contain distinct α (α_s and α_i) and common ß and γ subunits. Activation of either receptor type results in the exchange of GTP for bound GDP followed by dissociation of the G protein subunits. Although the mechanism by which α_s mediates stimulation of AC is well understood, less clear is the mechanism(s) involved in the inhibition of the enzyme by α_i. Several mechanisms may be involved in the inhibition. That is, inhibition may include: 1) the direct interaction of activated α_i with the catalytic subunit of the enzyme, or 2) ß+γ from G_i complexing with activated α_s, thus favoring the undissociated and inactive form of G_s. Evidence for both pathways has been reported (Katada et al., 1984a, b; Jakobs and Schultz, 1983; Roof et al., 1986). An alternative mechanism may involve prevention of stimulatory signal transduction at the receptor level. In this latter case, inhibition may be mediated by preventing the stimulatory receptor from forming a ternary complex composed of the

agonist, receptor and G_s protein. This complex appears to be a prerequisite for receptor mediated activation of the enzyme (DeLean et al., 1980; Kent et al., 1980; Gilman, 1987). Evidence supporting this mechanism has been recently reported by Romano et al. (1988; 1989). They showed that binding of ISO to the high affinity binding state of the ßAR was eliminated in the presence of PIA, an A_1-AdoR agonist. Although these data were interpreted to indicate that activation of the inhibitory A_1-AdoR prevented the ßAR from forming an agonist high affinity binding state (ternary complex), it can not be ascertained whether the inhibitory effect is mediated at the receptor or the G protein level. At the receptor level, inhibitory agonists could theoretically initiate alterations in the stimulatory receptor affecting agonist-receptor interaction or prevent the agonist-receptor complex from becoming active and thereby impairing signal transduction.

In 1987, Milecki et al. (1987) reported on the synthesis and partial characterization of an alkylating carbostyril derivative (C-Br). This compound and several of its congeners were shown to be potent ßAR agonists. In a subsequent study using membranes from rat reticulocytes, Standifer et al. (1989) showed that compared to ISO, C-Br was a full agonist and its interaction with the receptor was resistant to the effects of a guanine nucleotide. That is, the nonhydrolyzable guanine nucleotide derivative Gpp(NH)p only slightly reduced the ability of C-Br to inhibit [^{125}I]CYP binding (Standifer et

al., 1989). In addition, C-Br bound to the receptor in an irreversible manner and produced an antagonist-insensitive activation of AC activity. This suggested that C-Br is an irreversible agonist at the ßAR.

DDT cells have been shown to express both ßAR and A_1-AdoR. As shown in Chapter 3 of this dissertation and other reports (Ramkumar et al., 1990; Nordstedt and Fredholm, 1990; Ramkumar et al., 1991), ßAR mediated cAMP accumulation in DDT cells is inhibited by A_1-AdoR agonists. To obtain additional insight into the hypothesis that inhibition is mediated at the stimulatory receptor level, the ability of the A_1-AdoR to attenuate the cAMP response mediated by a permanently activated ßAR was investigated.

Results

A_1-AdoR Mediated Inhibitory Effects on ßAR-stimulated cAMP Accumulation by ISO and C-Br

Initial experiments were designed to determine the effect of CPA on ISO- and C-Br-stimulated cAMP accumulation in intact DDT cells. As shown in Figure 4-1, ISO and C-Br stimulated cAMP accumulation in a concentration-dependent manner with maximal effects of 29- and 32-fold above the basal level, respectively. The maximal responses to ISO and C-Br could not be readily compared because experiments were performed on different batches of cells. The EC_{50} value for C-Br was 0.2 nM and 2.7 nM for ISO. Both ISO and C-Br

stimulated cAMP accumulation in a concentration-dependent manner, however, in the presence of 1 μM CPA, the maximal response produced by ISO and C-Br was decreased by 86% and 75%, respectively. Figure 4-2 depicts the effect of CPA on cAMP accumulation stimulated by 10 μM ISO and 1 μM C-Br, concentrations which gave a maximal response. CPA attenuated the effect of ISO and C-Br in a concentration-dependent manner with the same potency (EC_{50} = 2.3 nM) and the same maximal response (89% inhibition).

Figure 4-3 illustrates the effect of the A_1-AdoR antagonist CPX on CPA mediated inhibition of ISO- and C-Br-stimulated cAMP accumulation. ISO (10 μM) and C-Br (1 μM) (concentrations which caused maximal stimulation of cAMP accumulation, see Figure 4-1) increased cAMP accumulation to a similar extent (27 and 28-fold above the basal level, respectively) and CPA (1 μM) inhibited their stimulation (81% for C-Br and 84% for ISO). CPX (5 μM), an A_1-AdoR antagonist, largely blocked the inhibitory effect of CPA, and the cAMP stimulations caused by C-Br and ISO were restored by 96% and 84%, respectively. This indicates that the inhibitory effect of CPA on C-Br and ISO-stimulated cAMP accumulation is A_1-AdoR mediated.

The effect of CPA on the basal level of cAMP in DDT cells is shown in Figure 4-4. CPA decreased the basal level of cAMP with an EC_{50} of 6.1 nM and a maximal inhibition of 82%.

Effect of CPA on the Insurmountable Component of C-Br-induced Stimulation

Figure 4-5 illustrates the time course of C-Br-induced increase in cAMP content and the effects of PROP and CPA. C-Br increased the cAMP content over a 10 min incubation period. After 3 min of incubation with C-Br, the addition of PROP (20 μM) had no effect on the subsequent rate of cAMP accumulation. In contrast, the addition of PROP and CPA (1 μM) 3 min after incubation with C-Br resulted in complete inhibition of further cAMP accumulation during the next 7 min of incubation. In fact, after addition of CPA, cAMP levels decreased.

The effect of CPA on the irreversible binding of C-Br to the ßAR is depicted in Figure 4-6. This is a representative Scatchard plot of specific [^{125}I]CYP binding after pretreatment of cells with C-Br, CPA and C-Br + CPA for 10 min at 37°C. CPA pretreatment did not alter the specific [^{125}I]CYP binding as compared to control (control, 54 pmol/ mg protein; CPA, 59 pmol/mg protein). After incubation of cells with 1 μM C-Br followed by 6 cell wash cycles, there was a 42% decrease in specific [^{125}I]CYP binding and this decrease was not affected by the presence of CPA during the 10 min preincubation period (C-Br, 28 pmol/mg protein; C-Br + CPA, 30 pmol/mg protein). There was also no change in the K_D value for [^{125}I]CYP among the 4 groups (control, 34 pM; C-Br, 32 pM; CPA, 25 pM; C-Br + CPA, 26 pM).

Figure 4-7 illustrates the effects of Gpp(NH)p and CPA on the concentration response of ISO to compete with [^{125}I]CYP for the ßAR binding site. ISO alone displaced specific [^{125}I]CYP binding with an IC_{50} of 60 nM and a Hill slope of 0.58. In the presence of Gpp(NH)p (100 µM), the displacement curve shifted to the right with an IC_{50} value of 406 nM and steepened with a Hill slope of 0.80. In contrast to Gpp(NH)p, CPA had no effect on either the IC_{50} value (70 nM) or the Hill slope (0.60) of the ISO displacement curve as compared with ISO alone.

Discussion

Receptor mediated activation of AC activity is a process with multiple steps that involve an agonist, receptor, a stimulatory guanine nucleotide binding protein (G_s) and the catalytic subunit of AC (Levitzki, 1987; Rodbell, 1980). General agreement now exists that agonists promote the formation of a ternary complex consisting of the agonist, receptor and G_s and this interaction accelerates the exchange of GDP for GTP at G_s. The binding of the agonist in the ternary complex is of high affinity. GTP destabilizes the ternary complex resulting in a decrease in agonist affinity and the release of an activated α_s subunit of G_s which directly stimulates the catalytic subunit of AC. Hydrolysis of GTP to GDP deactivates α_s and AC activity returns to the basal level (Gilman, 1987). In contrast to AC activation,

much less is known about the mechanism whereby receptor mediates inhibition of the enzyme. Similar to stimulatory receptors, inhibitory receptors are coupled to a guanine nucleotide binding protein (G_i) which dissociates into an α_i and ß+γ subunits in the presence of GTP. There is evidence supporting several inhibitory mechanisms which include 1) a direct interaction of α_i with the catalytic subunit of the AC (Katada et al., 1984b; Jakobs and Schultz, 1983; Roof et al., 1986), 2) ß+γ subunits released from G_i complexing with α_s to favor the inactive G_s complex (Katada et al., 1984a) and 3) prevention of stimulatory ternary complex formation (Romano et al., 1988; Romano et al., 1989).

In the present study, the effect of the inhibitory A_1-AdoR to attenuate the response of a permanently activated ßAR was investigated. Initial experiments were performed to partially characterize the actions of the irreversible ßAR agonist C-Br, using intact DDT cells. This compound was found to be a potent stimulator of cAMP accumulation, with a concentration required to produce half-maximal stimulation in the subnanomolar range. In addition, C-Br was a full ßAR agonist since it produced the same maximal response as the classical agonist, ISO. The potency and efficacy of C-Br using intact cells is consistent with a previous report on the effect of this compound to stimulate AC activity in isolated membranes from rat reticulocytes (Standifer et al., 1989).

Similar to ISO, C-Br stimulated cAMP accumulation in DDT cells was markedly attenuated by the selective A_1-AdoR agonist CPA. This attenuation was not reversed by high concentrations of either ßAR agonists but was largely blocked by the selective A_1-AdoR antagonist CPX. These data indicated that the inhibition of C-Br stimulated cAMP accumulation was mediated by an A_1-AdoR. Furthermore, the concentration of CPA to inhibit cAMP accumulation by equipotent and equieffective concentrations of ISO and C-Br was similar suggesting that activation of the ßAR by C-Br did not alter the the inhibitory effect of CPA.

In the experiments discussed above, the incubation time used was relatively short (6 min). Because the irreversible binding of C-Br to ßAR was time-dependent (Standifer et al., 1989), the responses observed at the end of short periods of incubation were probably due to both reversible and irreversible interactions of this agonist with the ßAR. To investigate the irreversible agonist effects of C-Br, the ßAR antagonist PROP was employed to compete off any C-Br which was reversibly bound to the ßAR. After a 3 min incubation with C-Br alone, the addition of an excess of PROP had no subsequent effect on the rate of cAMP formation as compared to that in the presence of C-Br alone. In contrast, when PROP and CPA were added after a 3 min incubation with C-Br alone, further accumulation of cAMP was inhibited. Although CPA attenuated C-Br stimulated cAMP accumulation, it did not affect the irreversible binding of C-Br to the ßAR. This

indicates that even though C-Br produced an antagonist-insensitive response once bound to the ßAR, activation of the inhibitory A_1-AdoR resulted in an attenuation of cAMP accumulation mediated by the permanently activated ßAR.

There is some evidence suggesting that one potential mechanism for receptor mediated inhibition of AC activity may involve signal transduction at the stimulatory receptor level. In 1978, Watanabe et al. reported that activation of the cardiac muscarinic inhibitory receptor decreased the affinity of ISO for the ßAR. Likewise, more recently, Romano et al. (1988; 1989) showed the A_1-AdoR agonist PIA reduced the affinity of ISO for the ßAR in rat cardiac membranes. This reduction in agonist affinity was similar to that observed in the presence of Gpp(NH)p (Romano et al., 1988; Romano et al., 1989). Because the guanine nucleotide sensitive agonist high affinity binding state of the receptor has been proposed to represent the ternary complex formation (Gilman, 1987), it was suggested that PIA prevented the stimulatory receptor from forming a ternary complex, a necessary prerequisite for the activation of AC. However, in the present study, CPA did not alter the interaction of ISO with the ßAR even though Gpp(NH)p did reduce agonist affinity. This indicated that CPA had no effect on ternary complex formation. The discrepancy between this observation and that reported by Romano et al. (1988) may be due to different cell types expressing different modes of inhibition. The inhibition of ternary complex formation may

be operative in cardiac cells whereas other inhibitory mechanisms may exist in DDT cells. Alternatively, during the process of DDT cell membrane preparation, the inhibitory effects on agonist binding may have become uncoupled.

The pathway of receptor activation initially involves a reversible interaction of the agonist (Ag) with the receptor (R) to form an Ag-R complex. The Ag-R complex then undergoes a conformational change to form an activated Ag-R complex which interacts with G_s. Although difficult to establish directly, there is some intriguing kinetic and structural evidence to suggest that the ßAR undergoes a conformational change induced by agonist (Contreras et al., 1986; Pedersen and Ross, 1985). An inhibitory mechanism could therefore involve preventing the Ag-R complex from becoming conformationally active. However, the data with the irreversible agonist are not consistent with this proposed mechanism. C-Br produced antagonist-insensitive activation of the ßAR suggesting that the Ag-R complex is in a permanently activated state, but the stimulation of cAMP accumulation is still inhibited by CPA. If CPA mediated inhibition involved inactivation of the ßAR, then it would be expected that CPA would have no effect on the irreversible component of C-Br-stimulated cAMP formation.

Data from the present study are also consistent with an inhibitory action beyond the receptor level. The basal cAMP accumulated in the absence of a ßAR agonist was also inhibited by CPA. Furthermore, in experiments not shown, CPA

inhibited forskolin-stimulated cAMP accumulation in DDT cells. Forskolin activates AC in a receptor-independent manner which may involve direct interaction with the catalytic subunit of the enzyme (Seamon and Daly, 1986). Although the mechanism for A_1-AdoR mediated inhibition of basal and forskolin-stimulated cAMP accumulation in DDT cells has not been fully delineated, evidence from other cell lines suggest that the subunits of G_i may be involved. Thus, α_i released by activation of the A_1-AdoR may directly attenuate the activity of the catalytic subunit of the enzyme, or the $\beta+\gamma$ subunits of G_i may complex with free α_s to form the inactive G_s complex (Katada et al., 1984a, b; Jakobs and Schultz, 1983; Roof et al., 1986). It is conceivable that inhibition may involve more than one mechanism acting in concert or a single mechanism may dominate depending on the mode of enzyme activation. Further studies will be necessary to elucidate these possibilities.

Over the past few years, significant effort has been expended in developing insurmountable receptor ligands as investigative tools (Posner et al., 1984; Homburger et al.,1984; Nelson et al., 1986; Baker et al., 1986) and more recently for therapeutics (Ullman and Svedmyr, 1988). These agents have the advantage of very slow dissociation rates from their receptors and hence may produce a long duration of action. In addition, once the drug-receptor complex is formed, the response will be independent of the plasma concentration which can be allowed to fall. This may

translate into a reduced incidence of side effects. In the case of agonists, the recently introduced ßAR agonist salmeterol has been shown to produce a sustained airway dilation probably due to an extremely tight binding to the ßAR (Brittain et al., 1981; Ullman and Svedmyr, 1988). On the other hand, one potential problem with slow dissociating agonists is modulating the response produced under conditions where the receptor is activated in an antagonist-insensitive manner. Therefore, administration of an antagonist may have no effect on the response. The data from the present study suggest that the sustained response mediated by C-Br can be controlled by activation of inhibitory receptor. Further development of agonists that produce sustained responses may need to take into account the interaction of inhibitory and stimulatory receptors to fully exploit their therapeutic potential.

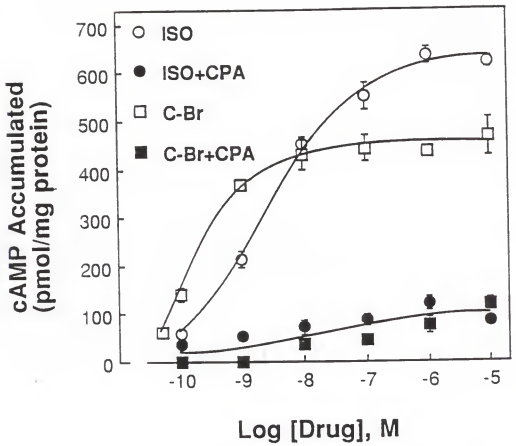

Figure 4-1. The effect of CPA on ISO- and C-Br-stimulated cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 100 µM rolipram, the indicated concentrations of ISO or C-Br, without or with 1 µM CPA for 6 min at 37°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The basal level of cAMP accumulated was 22±6, 14±3 pmol/mg protein/6 min for the ISO and C-Br experiments, respectively. Each data point is the mean±SE, n=4.

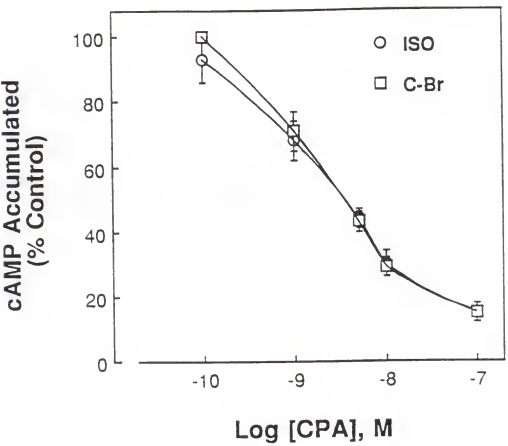

Figure 4-2. Inhibition of ISO- and C-Br-stimulated cAMP accumulation in DDT cells by CPA. Cells were incubated in HBSS containing 100 µM rolipram, 10 µM ISO or 1 µM C-Br and the indicated concentrations of CPA for 6 min at 37°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The control cAMP accumulated in the presence of ISO or C-Br alone was 646±22, 660±29 pmol/mg protein/6 min, respectively. The basal level of cAMP accumulated was 19±4 pmol/mg protein/6 min. Each data point is the mean±SE, n=4.

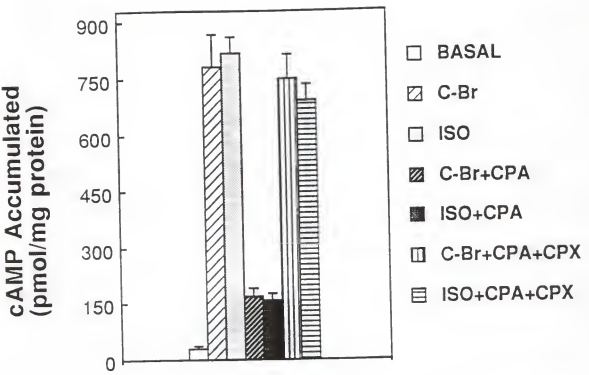

Figure 4-3. The effect of CPX on the inhibition of ISO- and C-Br-stimulated cAMP accumulation in DDT cells by CPA. Cells were incubated in HBSS containing 100 μM rolipram, 1 μM C-Br, 10 μM ISO, C-Br + 1 μM CPA, ISO + 1 μM CPA, C-Br + CPA + 5 μM CPX, or ISO + CPA + 5 μM CPX for 6 min at 37°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. Each data point is the mean±SE, n=4.

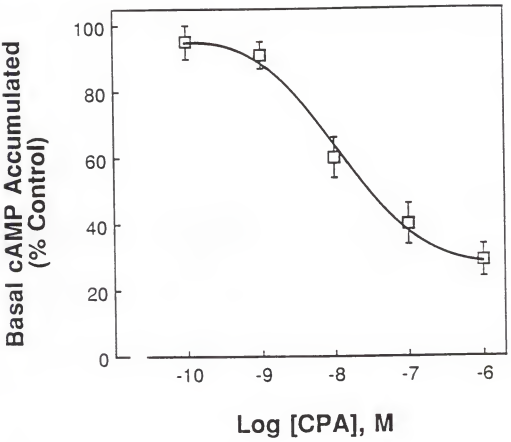

Figure 4-4. The effect of CPA on basal cAMP accumulation in DDT cells. Cells were incubated in HBSS containing 100 µM rolipram, without or with the indicated concentrations of CPA for 6 min at 37°C. At the end of the incubation period, the cAMP accumulated was determined as described in the "Methods" section. The basal control level of cAMP accumulated was 26±2 pmol/mg protein/6 min. Each data point is the mean±SE, n=4.

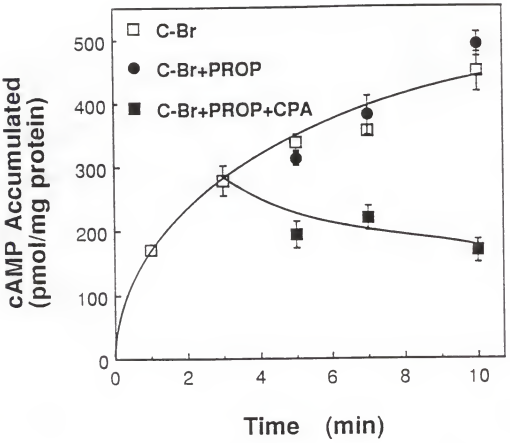

Figure 4-5. The effect of CPA on C-Br-stimulated cAMP accumulation in DDT cells. Cells were preincubated in HBSS containing 100 µM rolipram and 1 µM C-Br at 37°C. After 3 min of incubation, 20 µM propranolol or propranolol plus 1 µM CPA was added. At the times indicated, the cAMP content was determined as described in the "Methods" section. Each data point is the mean±SE, n=4.

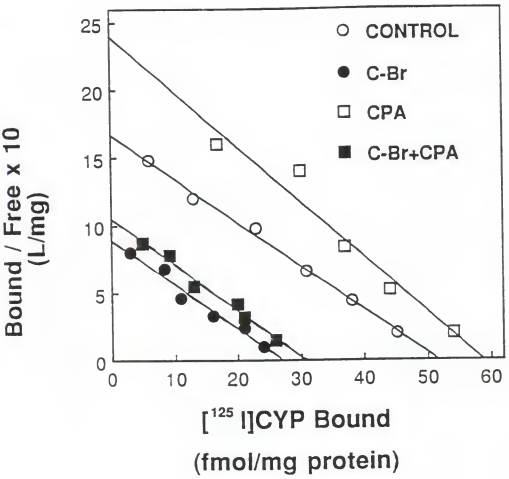

Figure 4-6. Scatchard plot of specific [^{125}I]CYP binding to DDT cell membranes after pretreatment with C-Br, CPA or C-Br+CPA. Cells were incubated in HBSS without or with 1 µM C-Br, 1 µM CPA or 1µM C-Br + 1 µM CPA for 10 min at 37°C. At the end of the incubation period, the cells were harvested and membranes prepared. Membrane protein was assayed with 6 - 100 pM [^{125}I]CYP as described in the "Methods" section. The data are plotted as the ratio of the amount of specifically bound ligand (pmol/mg protein) to free ligand (pmol/L) versus the amount of specifically bound ligand/mg protein. Data points are the mean of triplicate determination and are representative of 3 experiments.

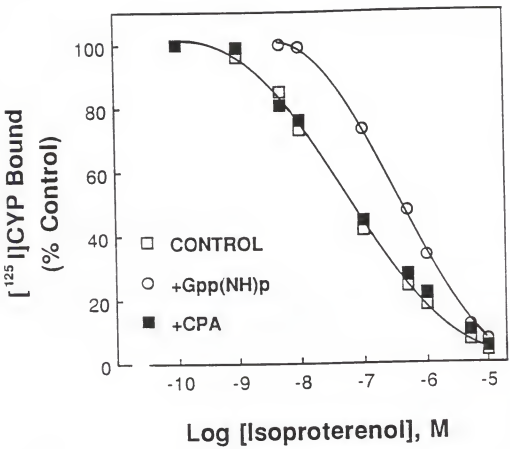

Figure 4-7. Inhibition of specific [^{125}I]CYP binding to DDT cell membrane by ISO in the presence of Gpp(NH)p or CPA. Membrane protein (50 µg) was incubated with 30 pM [^{125}I]CYP, the indicated concentrations of ISO, without or with 100 µM Gpp(NH)p or 1 µM CPA for 60 min. at 37°C. Data points are the mean of triplicate determination which varied by less than 6% and are representative of 3 experiments. The control specific [^{125}I]CYP binding was 53 fmol/mg protein.

LIST OF REFERENCES

Baker, S. P., Liptak, A. and Pitha, J. (1985) J. Biol. Chem. 260: 15820-15828

Baker, S. P., Sumners, C., Pitha, J. and Raizada, M. K. (1986) J. Neurochem. 47: 1318-1326

Belardinelli, L., Giles, W. R. and West, G. A. (1988) J. Physiol. (Lond.) 405: 615-633

Belardinelli, L., Isenberg, G. (1983) Am. J. Physiol. 224: H734-H737

Belardinelli, L and Lerman, B. B. (1990) Br. Heart J. 63: 3-4

Belardinelli, L., Linden, J. and Berne, R. M. (1989) Prog. Cardiovasc. Dis. 32(1): 73-97

Berne, R. M. (1963) Am. J. Physiol. 204: 317-322

Berne, R. M. (1986) News Physiol. Sci. 1: 163-167

Biaggioni, I., Killian, T. J., Mosqueda-Garcia, R., Robertson, R. M. and Robertson, D. (1991a) Circulation 83: 1668-1675

Biaggioni, I., Paul, S., Puckett, A. and Arzubiaga, C. (1991b) J. Pharmacol. Exp. Ther. 258(2): 588-592

Brittain, R. T., Dean, C. M. and Jack, D. (1981) Int. Encycl. Pharmacol. Ther. 104: 613-652

Brown, J. H. (1979) J. Cyclic Nucleotide Res. 5(6): 423-433

Bruns, R. F. (1990) Ann. N.Y. Acad. Sci. 603: 211-226

Bruns, R. F., Daly, J. W. and Snyder, S. H. (1980) Proc. Natl. Acad. Sci. USA 77(9): 5547-5551

Bruns, R. F., Fergas, J. H., Badger, E. W., Bristol, J. A., Santay, L. A., Hartman, J., Hays, S. J. and Huang, C. C. (1987) Naunyn-Schmiedebergs Arch. Pharmacol. 335: 59-63

Bruns, R. F., Lu, G. H. and Pugsley, T. A. (1986) Mol. Pharmacol. 29: 331-346

Contreras, M. L., Wolfe, B. B. and Molinoff, P. B. (1986) J. Pharmacol. Exp. Ther. 239: 136-143

Cooper, D. M. F. and Londos, C. (1979) J. Cyclic Nucleotide Res. 5: 289-302

Daly, J. W., Butts-Lamb, P. and Padgett, W. (1983) Cell. Mol. Neurobiol. 3: 69-80

Delahaba, G and Cantoni, G. L. (1959) J. Biol. Chem. 234: 603-608

Delahunty, T. M., Cronin, M. J. and Linden, J. (1988) Biochem. J. 255: 69-77

DeLean, A., Stadel, J. M. and Lefkowitz, R. J. (1980) J. Biol. Chem. 255: 7108-7117

Doss, R. C., Kramarcy, N. R., Harden, T. K. and Perkins, J. P. (1985) Mol. Pharmacol. 27: 507-516

Doss, R. C., Perkins, J. P. and Harden, T. K (1981) J. Biol. Chem. 256: 12281-12286

Drury, A. N. and Szent-Gyorgyi, A. (1929) J. Physiol. (Lond.) 68: 213-237

Endoh, M., Maruyama, M. and Iijima, T. (1985) Am. J. Physiol. 249: H309-H320

Endoh., M., Muruyama, M. and Taira, N. (1983) Physiology and Pharmacology of Adenosine Derivatives, ed. by Daly, J. W., Kuroda, Y. and Philis, J. W., New York: Raven Press, pp 127-141

Fain, J. N. and Malbon, C. C. (1979) Mol. Cell Biochem. 23: 1-27

Fastbom, J. and Fredholm, B. B. (1990) Neuroscience 34: 759-769

Fredholm, B. B., Lindgren, E. and Lingstrom, K. (1985) Br. J. Pharmacol. 86: 509-513

Gavish, M., Goodman, R. R. and Synder, S. H. (1982) Science 215: 1633-1635

Gerlach, E., Deuticke, B. and Dreisbach, R. H. (1963) Naturwissenschaft 50: 228-229

Gerwins, P. and Fredholm, B. B. (1991) Mol. Pharmacol. 40: 149-155

Gerwins, P., Nordstedt, C. and Fredholm, B. B. (1990) Mol. Pharmacol. 38: 660-666

Gilman, A. G. (1987) Ann. Rev. Biochem. 56: 615-649

Goodman, R. R., Cooper, M. J., Gavish, M. and Snyder, S. H. (1982) Mol. Pharmacol. 21: 329-335

Green, A. (1987) J. Biol. Chem. 262: 15702-15707

Gunst, S. J., Stropp, J. Q. and Flavahan, N. A. (1989) J. Appl. Physiol. 67(3): 1294-1298

Harden, T. K. (1983) Pharmacol. Rev. 35 (1): 5-32

Hazeki, O. and Ui, M. (1981) J. Biol. Chem. 256(6): 2856-2862

Heisler, S., Desjardins, D. and Nguyen, M. H. (1985) J. Pharmacol. Exp. Ther. 232(1): 232-238

Hill, A. V. (1913) Biochem. J. 7: 471-480

Hill, S. J. and Kendall, D. A. (1987) Br. J. Pharmacol. 91: 661-669

Hollingsworth, E. B. and Daly, J. W. (1985) Biochim. Biophys. Acta 847: 207-216

Homburger, V., Pantaloni, C., Lucas, M., Gozlan, H. and Bockaert, J. (1984) J. Cell. Physiol. 121: 589-597

Hutchison, A. J., Webb, R. L., Oei, H. H., Ghai, G. R., Zimmerman, M. B. and William, M. (1989) J. Pharmacol. Exp. Ther. 251: 47-55

Hutchinson, A. J., Williams, M., Jesus, R. D., Yokoyama, R., Oei, H. H., Ghai, G. R., Webb, R. L., Zoganas, H. C., Stone, G. A. and Jarvis, M. F. (1990) J. Med. Chem. 33: 1919-1924

Jacobson, K. A., Ukena, D., Kirk, K. L. and Daly, J. W. (1986) Proc. Natl. Acad. Sci. USA 83: 4089-4093

Jakobs, K. H. and Schultz, G. (1983) Proc. Natl. Acad. Sci. USA 80: 3899-3902

Jarvis, F. M., Schulz, R., Hutchison, A. J., Do, U. H., Sills, M. A. and Williams, M. (1989) J. Pharmacol. Exp. Ther. 251(3): 888-893

Jarvis, F. M. and Williams, M. (1989) Eur. J. Pharmacol. 168: 243-246

Katada, T., Bokoch, G. M.,Smigel, M. D., Ui, M. and Gilman, A. G. (1984a) J. Biol. Chem. 259: 3586-3595

Katada, T., Northup, J. K., Bokoch, G. M., Ui, M. and Gilman, A. G. (1984b) J. Biol. Chem. 259: 3578-3585

Kendall, D. A. and Hill, S. J. (1988) J. Neurochem. 50: 497-502

Kenimer, J. G. and Nirenberg, M. (1981) Mol. Pharmacol. 20: 585-591

Kent, R. S., DeLean, A. and Lefkowitz, R. J. (1980) Mol. Pharmacol. 17: 14-23

Klotz, K. N. and Lohse, M. J. (1986) Biochem. Biophys. Res. Commun. 140: 406-413

Kolassa, N., Pfleger, K. and Rummel, W. (1970) Eur. J. Pharmacol. 9: 265-268

Kurachi, Y., Nakajima, T. and Sugimoto, T. (1986) Pflugers Arch. 407: 264

Lad, P. M., Nielsen, T. B., Londos, C., Preston, M. S. and Rodbell, M. (1980) J. Biol. Chem. 255: 10841-10846

Levitzki, A. (1987) Biochem. Pharmacol. 27: 2083-2088

Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M. J., Dumont, J. E. and Vassart, G. (1989) Science 244: 569-572

Liles, W. C. and Nathanson, N. (1986) J. Neurochem. 46: 89-95

Linden, J. (1991) FASEB J. 5: 2668-2676

Linden, J. and Delahunty, T. M. (1989) Trends Pharmacol. Sci. 10: 114-120

Linden, J., Patel, A. and Sadek, S. (1985) Circ. Res. 56: 279-284

Lindner, F. and Rigler, R. (1931) Pfluegers Arch. 226: 697-708

Lohse, M. J., Klotz, K. N., Schwabe, U., Cristalli, G., Vittori, S. and Griffantini, M. (1988) Naunyn-Schmiedeberg's Arch. Pharmacol. 337: 687-689

Lohse, M. J., Lenschow, V., Schwabe, U. (1984) Mol. Pharmacol. 26: 1-9

Londos, C., Cooper, D. M. F. and Wolff, J. (1980) Proc. Natl. Acad. Sci. USA 77: 2551-2554

Londos, C. and Wolff, J. (1977) Proc. Natl. Acad. Sci. USA 74: 5482-5486

Londos, C., Wolff, J. and Cooper, D. M. F. (1983) Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides, ed by Baer, H. P. and Drummond, G. I., New York: Raven Press, pp 271-281

Londos, C., Wolff, J. and Cooper, D. M. F. (1981) Purinergic Receptors, ed by Burnstock, G., London: Chapman and Hall, pp 287-323

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193: 265-275

Lupica, C. R., Cass, W. A., Zahniser, N. R. and Dunwiddie, T. V. (1990) J. Pharmacol. Exp. Ther. 252(3): 1134-1141

Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentier, M., Vanderhaegen, J. J., Dumont, J. E., Vassart, G. and Schiffmann, S. (1990) Biochem. Biophys. Res. Commun. 173: 1169-1178

Malbon, C. C., Moreno, F. J., Cabelli, R. J. and Fain, J. N. (1978) J. Biol. Chem. 253: 671-678

Martens, D. M., Lohse, J., Rauch, B. and Schwabe, U. (1987) Naunyn-Schmiedeberg's Arch. Pharmacol. 336: 342-348

Milecki, J., Baker, S. P., Standifer, K. M., Ishizu, T., Chida, Y., Kusiak, J. W. and Pitha, J. (1987) J. Med. Chem. 30: 1563-1566

Mills, I. and Gewirtz, H. (1990) Biochem. Biophys. Res. Commun. 168(3): 1297-1302

Mitsuhashi, M. and Payan, D. G. (1988) J. Cellular Physiol. 134: 367-375

Morgan, N. G. (1989) Cell Signalling, ed. by Morgan, N. G., New York: Guilford Press, pp. 63-66

Munshi, R. and Linden, J. (1989) J. Biol. Chem. 264: 14853-14589

Nakata, H. (1989a) J. Biol. Chem. 264: 16545-16551

Nakata, H. (1989b) Mol. Pharmacol. 35: 780-786

Nathanson, N. M., Klein, W. L. and Nirenberg, M. (1978) Proc. Natl. Acad. Sci. USA 75(4): 1788-1791

Nazarea, M., Okajima, F. and Kondo, Y. (1991) Eur. J. Pharmacol.- Mol. Pharmacol. Section 206: 47-52

Nelson, C. A., Muther, T. F., Pitha, J. and Baker, S. P. (1986) J. Pharmacol. Exp. Ther. 237: 830-836

Nickerson, M. (1956) Nature (Lond.) 178: 697-698

Norris, J. S., Cramer, D. J., Brown, F., Popovich, K. and Cornett, L. E. (1983) J. Recept. Res. 3: 623-645

Norris, J. S. and Kohler, P. O. (1974) Nature (Lond.) 248: 422-424

Norris, J. S. and Kohler, P. O. (1977) Endocrinology 100: 613-618

Ohisalo, J. J. and Stouffer, J. E. (1979) Biochem. J. 178: 249-251

Olsson, R. A. and Pearson, J. D. (1990) Physiol. Rev. 70(2): 761-845

Paterson, A. R. P. and Oliver, J. M. (1971) Can. J. Biochem. 49: 271-274

Pedersen, S. E. and Ross, E. M. (1986) J. Biol. Chem. 260(26): 14150-14157

Pelleg, A. and Porter, S. R. (1990) Pharmacotherapy 10(3): 157-174

Pittman, R. N., Reynolds, E. E. and Molinoff, P. B. (1984) J. Pharmacol. Exp. Ther. 230(3): 614-618

Posner, P., Peterson, C. F., Pitha, J. and Baker, S. P. (1984) Eur. J. Pharmacol. 100: 373-376

Ramkumar, V., Barrington, W. W., Jacobson, K. A. and Stiles, G. L. (1990) Mol. Pharmacol. 37: 149-156

Ramkumar, V., Olah, M. E., Jacobson, K. A. and Stiles, G. L. (1991) Mol. Pharmacol. 40: 639-647

Ramkumar, V., Pierson, G. and Stiles, G. L. (1988) Prog. Drug Res. 32: 195-247

Ramkumar, V.and Stiles, G. L.(1988) J. Pharmacol. Exp. Ther. 246: 1194-1200

Rapiejko, P. J. and Malbon, C. C. (1987) Biochem. J. 241: 765-771

Rodbell, M. (1980) Nature 284: 17-22

Romano, F. D., Fenton, R. A. and Dobson, J. G. (1988) Second Messengers Phosphoproteins 12(1): 29-43

Romano, F. D., Macdonald, S. G. and Dobson, J. G. (1989) Am. J. Physiol. 257: H1088-H1095

Ronnett, G. V. and Lane, M. D. (1981) J. Biol. Chem. 256: 4704-4707

Roof, D. J., Applebury, M. L., Sternweis, P. C. (1985) J. Biol. Chem. 260: 16242-16249

Sattin, A. and Rall, T. W. (1970) Mol. Pharmacol. 6: 13-23

Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51: 660-670

Schubert, P. (1985) Adenosine: Receptor and Modulation of Cell Function, ed. by Stefanovich, V., Rudolphi, K. and Schubert, P., Oxford, England: IRL Press Limited, pp. 117-129

Seamon, K. B. and Daly, J. W. (1986) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 20: 1-50

Sevilla, N., Tolkovsky, A. M. and Levitzki, A. (1977) FEBS Lett. 81: 339-341

Shimizu, H. and Daly, J. W. (1970) Biochem. Biophys. Acta. 22: 465-473

Shryock, J., Patel, A., Belardinelli, L. and Linden, J. (1989) Am. J. Physiol. 256: H321-H327

Shryock, J., Travagli, H. C. and Belardinelli, L. (1992) J. Pharmacol. Exp. Ther. (accepted for publication)

Standifer, K. M., Pitha, J. and Baker, S. P. (1989) Naunyn-Schmiedeberg's Arch. Pharmacol. 339: 129-137

Stephenson, R. P. (1956) Br. J. Pharmacol. 11: 379-393

Stiles, G. L. (1985) J. Biol. Chem. 260: 6728-6732

Stiles, G. L., Daly, D. J. and Olsson, R. A. (1985) J. Biol. Chem. 260: 10806-10811

Stroher, M., Nanoff, C. and Schutz, W. (1989) Naunyn-Schmiedeberg's Arch. Pharmacol. 340: 87-92

Stryer, L. and Bourne, H. R. (1986) Ann. Rev. Cell Biol. 2: 391-419

Toews, M. L. (1987) Mol. Pharmacol. 31: 58-68

Trivedi, B. K., Bridges, A. J. and Bruns, R. F. (1990) Adenosine and Adenosine Receptors, ed. by Williams, M., Clifton, New Jersey: The Humana Press Inc. pp 57-103

Trost, T. and Schwabe, U. (1981) Mol. Pharmacol. 19: 228-235

Trussel, L. O. and Jackson, H. B. (1985) Proc. Natl. Acad. Sci. USA 82: 4857-4861

Ueeda, M., Thompson, R. D., Arroyo, L. H. and Olsson, R. A. (1991) J. Med. Chem. 34(4): 1340-1344

Ullman, A. and Svedmyr, N. (1988) Thorax 43: 674-678

Van Calker, D., Miller, M. and Hamprecht, B. (1979) J. Neurochem. 33: 999-1005

Watanabe, A. M., McConnaughey, M. M., Strawbridge, R. A., Fleming, J. W., Jones, L. R. and Besch, H. R. (1978) J. Biol. Chem. 253: 4833-4836

Wesley, R. C. and Belardinelli, L. (1989) Circulation 80: 128-137

Williams, M. and Risley, E. A. (1980) Proc. Natl. Acad. Sci. USA 77: 6892-6896

Wolff, J., Londos, C. and Cooper, D. M. F. (1981) Adv. Cyclic Nucleotide 14: 199-214

Wu, S. N., Linden, J., Visentin, S., Boykin, M. and Belardinelli, L. (1989) Circ. Res. 65(4): 1066-1076

Xu, D., Kong, H. and Liang, B. T. (1992) Circ. Res. 70(1): 56-65

Yeung, S. M. and Green, R. D. (1983) J. Biol. Chem. 258: 2334-2339

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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